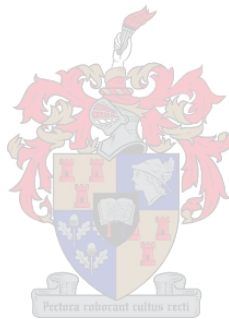

Genotypic and epidemiological characterization of Methicillin resistant *Staphylococcus aureus* (MRSA) and Coagulase Negative Staphylococcal (CoNS) strains isolated at Tygerberg Hospital

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Thesis presented in fulfilment of the requirements for the degree of Master of Science in the
Faculty of Medicine and Health Sciences at Stellenbosch University.



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December 2016

Declaration

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Abstract

Background

The global burden of methicillin resistant *Staphylococcus aureus* (MRSA) can be largely attributed to *S. aureus*' ability to acquire the resistance element, SCC*mec* (Staphylococcal chromosome complex *mec*). Classification of SCC*mec* types is an essential component of staphylococcal epidemiology and is based on the arrangement and classes of the *mec* and *ccr* gene complexes and Open Reading Frames (ORFs) in the joining (J) regions. At least twelve SCC*mec* types and numerous subtypes have been described to date. We identified potentially novel and novel variant SCC*mec* types in MRSA isolates from a tertiary hospital in Cape Town, South Africa. This study aimed to describe the molecular structure and possible origin of these novel elements in our setting, and to determine the prevalence of these SCC*mec* types.

Methods

We screened 87 clinical MRSA and 100 methicillin resistant coagulase negative *Staphylococci* (MR-CoNS) isolates using a multiplex PCR for SCC*mec* typing. Additional typing employed a combination of six multiplex PCRs on 3 MRSA isolates each from the novel and novel variant types. Whole genome sequencing (WGS) was performed on representative isolates using the Illumina next-generation sequencing platform.

Results

Among the MRSA isolates, 36% contained the novel SCC*mec* type (*ccrC*/Class A *mec*), followed by SCC*mec* IV (24%), the novel variant SCC*mec* type (*ccrA1B1*, *ccrC*/Class B *mec*) (17%) and SCC*mec* III (11%). Only one MR-CoNS isolate contained the novel type. Preliminary genomic analysis support the PCR findings, and show the presence of a possible truncated SCC_{Hg} element in the novel, novel variant and SCC*mec* III isolates.

Discussion

We have successfully optimized and implemented two SCC*mec* typing assays on MRSA and MR-CoNS isolates in our setting and in doing so, identified possible novel and novel variant SCC*mec* elements. The novel SCC*mec* type is common among local MRSA isolates, and may reflect clonal spread within the hospital. Preliminary WGS analysis showed that these isolates are composites of SCC_{Hg} and SCC*mec* elements, however further phylogenetic analysis is required to provide insights into how these elements emerged.

Opsomming

Agtergrond

Die wêreldwye las van metisillienweerstandige *Staphylococcus aureus* (MRSA) kan toegeskryf word aan *S. aureus* se vermoë om die antibiotiese weerstandbiedende element, “*Staphylococcal chromosome complex mec*” (SCC*mec*) op te neem. Klassifikasie van SCC*mec* is ‘n essensiële komponent in die epidemiologiese ondersoek van stafilokokkus-infeksies en is gebaseer op die kombinasies van verskillende “*mec*” en “*ccr*” geenkomplekse en oopleesrame (OLR) in die “*joining (J) regions*” van die element. Ten minste twaalf SCC*mec*-tipes en verskeie subtypes is al beskryf en gepubliseer en kom in beide MRSA en metisillienweerstandige koagulase negatiewe stafilokokke (MR-KoNS) voor. Ons het ‘n potensiële nuwe SCC*mec*-tipe en variant tipe in MRSA-isolate van Tygerberg Akademiese Hospitaal ontdek. In hierdie studie het ons gepoog om die molekulêre struktuur, moontlike oorsprong en voorkoms van die nuwe tipe SCC*mec*-isolate in hierdie instelling te ondersoek.

Metodes

‘n veelvoudige polimerasekettingreaksie (vPKR) is op 87 MRSA en 100 MR-KoNS-isolate uitgevoer. Verdere epidemiologiese ondersoek is vir drie isolate elk van die nuwe tipe en nuwe variant tipe gedoen met ‘n protokol wat die gebruik van ses mPKR-reaksies insluit. Heel-genoomvolgordebepaling is op verteenwoordigende isolate met Illumina tegnologie gedoen.

Resultate

Ses-en-dertig persent van die MRSA isolate het die nuwe SCC*mec* tipe (*ccrC*/Klas A *mec*) bevat, gevolg deur SCC*mec* IV (24%), die nuwe variant (*ccrA1B1*, *ccrC*/Class B *mec*) (17%) en SCC*mec* III (11%). Slegs een MR-KoNS-isolaat het die nuwe tipe bevat. Voorlopige analise van die genome van hierdie nuwe tipes ondersteun die PKR resultate, en dui op die voorkoms van ‘n verkorte SCC_{Hg} element in die nuwe, variant en SCC*mec* III-isolate.

Bespreking

Ons het twee SCC*mec*-tiperingsmetodes in ons instelling suksesvol geïmplementeer en geïmplementeer op MRSA- en MR-KoNS-isolate en daardeur ‘n nuwe SCC*mec*-tipe en nuwe variant SCC*mec*-tipe ontdek. Die nuwe SCC*mec* tipe is algemeen in die plaaslike MRSA-isolate en dit mag ‘n aanduiding wees dat hierdie tipe op ‘n klonale wyse versprei in die hospitaal. Heel-genoomvolgordebepalings-analise wys daarop dat hierdie nuwe elemente samestellings van

SCC_{Hg} en SCC_{mec} elemente is, maar verdere filogenetiese ondersoeke moet uitgevoer word, om insig te gee oor die oorsprong en verspreiding van hierdie nuwe element.

Table of Contents

Declaration	i
Abstract	ii
Opsomming	iii
Table of Contents	v
Acknowledgements	vi
List of Abbreviations	vii
List of Tables	x
List of Figures	xi
Chapter 1: Literature Review	1
1.1 Introduction	1
1.2 Methicillin resistant <i>Staphylococcus aureus</i> (MRSA)	1
1.3 SCCmec	5
1.4 Molecular typing methods	16
1.5 MR-CoNS	18
1.6 Problem statement	21
Aims and Objectives	22
Chapter 2: The SCCmec typing of MRSA and MR-CoNS isolates according to the method of Milheirico et al. 2007	23
2.1 Introduction	23
2.2 Methods	24
2.3 Results	30
2.4 Discussion	38
Chapter 3: The SCCmec typing of MRSA and MR-CoNS isolates according to the method of Kondo et al. 2007	43
3.1 Introduction	43
3.2 Methods	44
3.3 Results	48
3.4 Discussion	56
Chapter 4: Whole Genome Sequencing	59
4.1. Introduction	59
4.2. Methods	60
4.3. Results	71
4.4. Discussion	81
Chapter 5: Conclusion	85
Addendum A: Materials	88
Addendum B: Supplementary Methods	90
References	92

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List of Abbreviations

ACME	Arginine catabolic mobile element
ACT	Artemis Comparison Tool
AST	Antimicrobial susceptibility test
ATCC	American Type Culture Collection
bGLU	β -Glucosidase test
<i>ccr</i>	cassette chromosome recombinase
CDC	Centres for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase negative staphylococcus/staphylococci
CPGR	Centre for Proteomic and Genomic Research
dNTPs	Deoxyribonucleotide triphosphates
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
FOX	Cefoxitin
gDNA	Genomic deoxyribonucleic acid
HGT	Horizontal gene transfer
indel	Insertions and deletions
IWG SCC	International Working Group for SCC <i>mec</i>
J region	Joining region
MGE	Mobile genetic element
MLST	Multi locus sequence typing
mPCR	Multiplex polymerase chain reaction
MR-CoNS	Methicillin resistant coagulase negative staphylococcus/staphylococci

MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
CA-MRSA	Community associated methicillin resistant <i>Staphylococcus aureus</i>
HA-MRSA	Hospital associated methicillin resistant <i>Staphylococcus aureus</i>
LA-MRSA	Livestock associated methicillin resistant <i>Staphylococcus aureus</i>
MRSE	Methicillin resistant <i>Staphylococcus epidermidis</i>
MSA	Mannitol salt agar
MSSA	Methicillin sensitive <i>Staphylococcus aureus</i>
NTC	No template control
NGS	Next-generation sequencing
NHLS	National Health Laboratory Service
NICU	Neonatal intensive care unit
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine leucocidin
QC	Quality control
RAST	Rapid Annotation using Subsystem Technology
RATT	Rapid Annotation Transfer Tool
SBS	Sequencing-by-synthesis
SCC _{Hg}	Staphylococcal cassette chromosome mercury
SCC _{mec}	Staphylococcal cassette chromosome <i>mec</i>
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
SPAdes	St Petersburg Genome Assembler

ssDNA	Single stranded deoxyribonucleic acid
SSTI	Skin and soft tissue infections
ST	Sequence type
TAE	Tris-acetic acid-ethylenediaminetetraacetic acid buffer
TE	Tris-ethylenediaminetetraacetic acid
T _m	Melting temperature
TSB	Tryptic soy broth
VISA	Vancomycin intermediate <i>Staphylococcus aureus</i>
VRSA	Vancomycin resistant <i>Staphylococcus aureus</i>
WGS	Whole genome sequencing

List of Tables

Chapter 1: Literature Review	1
Table 1.1 Currently identified <i>ccr</i> gene complexes	9
Table 1.2 Currently identified <i>mec</i> gene complexes	11
Table 1.3 Additional resistance elements that may be found in the SCC <i>mec</i> element	12
Table 1.4 The twelve currently published SCC <i>mec</i> types (as of August 2016)	13
Chapter 2: The SCC<i>mec</i> typing of MRSA and MR-CoNS isolates according to the method of Milheirico et al. 2007	23
Table 2.1 List of primers used for the Milheirico (2007) protocol	28
Table 2.2 Reference strains used in the Milheirico (2007) protocol	29
Chapter 3: The SCC<i>mec</i> typing of MRSA and MR-CoNS isolates according to the method of Kondo et al. 2007	43
Table 3.1 Reference strains (controls) used in the Kondo protocol	44
Table 3.2 List of primers used for the Kondo (2007) protocol	46
Table 3.2 List of primers used for the Kondo (2007) protocol (continued)	47
Chapter 4: Whole Genome Sequencing	59
Table 4.1 Representative samples chosen for WGS	60
Table 4.2 Barcodes (indices) applied during WGS	64
Table 4.3 Assembly statistics for all isolates	73
Addendum A	88
Table A1 Buffer compositions	88

List of Figures

Chapter 1: Literature Review	1
Figure 1.1 The base structure of the SCC <i>mec</i> element (adapted from http://www.staphylococcus.net).....	7
Figure 1.2 Specific SCC <i>mec</i> structures of types I to VIII (Source: Ito et al. 2009).....	8
Chapter 2: The SCC<i>mec</i> typing of MRSA and MR-CoNS isolates according to the method of Milheirico et al. 2007	23
Figure 2.1 A MR-CoNS isolate as determined by the cefoxitin disk diffusion test. The arrow shows how the zone of inhibition zone was measured.	30
Figure 2.2 Species variation of 100 MR-CoNS samples. Species were identified using the VITEK®2 (bioMérieux, South Africa) automated system. The majority of the isolates were identified as <i>S. epidermidis</i> , <i>S. haemolyticus</i> and <i>S. hom. Hominis</i> . The prevalence of <i>S. warneri</i> , <i>S. saprophyticus</i> and <i>S. capitis</i> were very low. Five isolates could not be identified.....	31
Figure 2.3 SCC <i>mec</i> typing. The results of molecular SCC <i>mec</i> typing on 87 MRSA isolates are shown. The majority of the isolates belonged to the N (orange), type IV (green) and NV (purple) groups. Types III and II (red and blue respectively) had lower prevalence and only one type V isolate was found. No SCC <i>mec</i> type I or VI isolates were identified. 5% of the isolates were untypeable by current methods (yellow).	32
Figure 2.4 Milheirico mPCR. A comparison between two SCC <i>mec</i> type V reference strains, BAA-1688 and WIS, and a Type V variant isolate from our study. A DNA marker (ladder) and NTC were included.	33
Figure 2.5 Milheirico mPCR results. Six controls and a number of representative isolates are shown in this summary gel, along with a NTC (no template control) and DNA markers (Ladders) to estimate band size.	34

Figure 2.6	Milheirico mPCR on MR-CoNS isolates: A. Six controls and five isolates are shown, including DNA markers (Ladders); B. Additional isolates from the same PCR are shown, along with DNA markers (Ladders) and an NTC (no template control).	36
Chapter 3: The <i>SCCmec</i> typing of MRSA and MR-CoNS isolates according to the method of Kondo et al. 2007		43
Figure 3.1	Kondo mPCR 1. <i>SCCmec</i> types I to VI were used as controls. The novel and novel variant isolates and representative strains for each control were included. DNA markers (Ladders) and an NTC were also included.	48
Figure 3.2	Kondo mPCR 2. <i>SCCmec</i> types I to V were used as controls. The novel and novel variant isolates and representative strains for each control were included. DNA markers (Ladders) and an NTC were also included.	49
Figure 3.3	Kondo mPCR 3. <i>SCCmec</i> types I and three subtypes of IV were the control strains in this mPCR. The novel and novel variant isolates and representative strain for control IV were included. DNA markers (Ladders) and an NTC were also included.	51
Figure 3.4	Kondo mPCR 4. Four subtypes of <i>SCCmec</i> type II were used as controls in this mPCR, along with type III and V. A DNA marker (Ladder) and NTC were also included.	52
Figure 3.5	Kondo mPCR 5. <i>SCCmec</i> types II and III were the control strains in this mPCR. The novel and novel variant isolates and representative strains for each control were included. DNA markers (Ladders) and an NTC were also included.	53
Figure 3.6	Kondo mPCR 6. <i>SCCmec</i> types II and III were the control strains in this mPCR. The novel and novel variant isolates and representative strains for	

	each control were included. DNA markers (Ladders) and an NTC were also included.....	54
Chapter 4: Whole Genome Sequencing		59
Figure 4.1	Formation of clonal clusters for Illumina sequencing (http://www.illumina.com)	65
Figure 4.2	Sequencing by synthesis technology used by the MiSeq sequencer. Bases are sequentially washed over the flow cell, and only one matching nucleotide can be incorporated at a time depending on the sequence of the strand. When this happens, a fluorescent signal is emitted and captured by the sequencer. (http://www.illumina.com).....	66
Figure 4.3	Genome assembly and annotation pipeline used for this study.....	68
Figure 4.4	Quality scores (Q) across all reads.....	72
Figure 4.5	QUAST quality report (sample TKB-106). All statistics are based on contigs >500 bp, unless stated otherwise.	73
Figure 4.6	Visual comparison of a reference sequence (JKD 6008, top) and ordered contigs (TKB-105, bottom). Red blocks show similarity/synteny between the genomes, blue blocks show areas of synteny that have an inversed configuration in the query sequence. White areas are sequences that are unique to either the reference or query.	75
Figure 4.7	Alignment of the <i>SCC_{mec}</i> regions of the reference (JKD6008, top) and query (TKB-105, bottom). The arrows below the figure indicate regions unique to the query, where no annotation was transferred.	77
Figure 4.8	Structural comparison between a reference <i>SCC_{mec}</i> type III and two <i>SCC</i> types found in our study. A: <i>SCC_{Hg}</i> and <i>SCC_{mec}</i> III (commonly carried together). B: <i>SCC_{mec}</i> IIIA. C: Novel <i>SCC</i> element (designated Ψ SCC).	78

Figure 4.9 Structural comparison between a reference *SCCmec* type I and a novel variant SCC element found in our study. A: *SCCmec* I. B: Novel variant.... 80

Addendum A	88
Figure A1 KAPA Universal DNA Ladder.....	89
Figure A2 NovelJuice Tracking Dyes	89

Chapter 1: Literature Review

1.1 Introduction

Staphylococcus aureus (*S. aureus*) is a commensal microorganism that forms part of naturally occurring flora in the anterior nares of approximately 30% of the human population and yet, it is one of the leading causes of bacterial infection worldwide (DeLeo et al. 2010). Unlike most other members of its genus, *S. aureus* is coagulase positive, giving it the ability to cause blood clotting by converting fibrinogen to fibrin. Fibrin clots protect against phagocytosis and other host defenses and consequently contributes to its virulence (McAdow et al. 2012). *Staphylococcus aureus* is the most pathogenic member of its genus (Shore & Coleman 2013), with infections that range from minor skin ailments to serious and often fatal systemic infections such as skin and soft tissue infections (SSTI) and necrotizing pneumonia (DeLeo et al. 2010). Staphylococcal infections occur in patients from all demographic descriptions, with disease outcomes dependent on the interplay between bacterial virulence and the host's immune response (Shore & Coleman 2013). Furthermore, *S. aureus* has the ability to rapidly acquire resistance, in particular to the β -lactam class of antibiotics; this class includes penicillin as well as methicillin and the cephalosporins.

1.2 Methicillin resistant *Staphylococcus aureus* (MRSA)

1.2.1 Overview

Staphylococcus aureus can be divided into two clinically significant groups, methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA). Methicillin is a member of the β -lactam family of antibiotics, which have a high affinity for penicillin-binding-proteins (PBPs) in the cell wall. When PBPs are bound by the β -lactams, peptidoglycan synthesis is interrupted, leading to cell death. MSSA isolates may develop resistance to β -lactams by the acquisition of a

specific large mobile genetic element (MGE), known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama et al. 2000). SCC*mec* carries the *mecA* gene, which encodes an altered PBP known as PBP2a or PBP2'. The PBP2a is a 78-kDa class B transpeptidase that catalyses the formation of cross-bridges in bacterial cell wall peptidoglycan. It has a very low binding affinity for β -lactam antibiotics (Hanssen & Ericson Sollid 2006) and as a result peptidoglycan synthesis will continue even in the presence of β -lactams and the cell will survive.

1.2.2 History of MRSA

Within two years of the introduction of penicillin in 1941, resistance was reported in *S. aureus*. Shortly after, the first description of penicillinase (penicillin hydrolyzing enzymes) producing *S. aureus* strains was made (Chambers 2001). In 1959, the first penicillinase stable penicillin, methicillin, was discovered (Jevons 1961). Methicillin-resistant *S. aureus* was first identified, one year after the introduction of the semisynthetic penicillin, methicillin, in patients who were recently admitted to a healthcare facility or who underwent surgery. These infections were termed healthcare associated MRSA (HA-MRSA) (Chongtrakool et al. 2006; DeLeo et al. 2010). However, in the 1990s MRSA was observed in patients who had none of the typical risk factors such as recent hospital admission, residence in a nursing home or other conditions which may have caused exposure to healthcare facilities or MRSA infected persons (Huang et al. 2006). These patients displayed SSTIs as well as necrotizing pneumonia and this was termed community associated MRSA (CA-MRSA) (Barbier et al. 2010). Initially, patients with CA-MRSA infections had case similarities and many cases were identified based on specific molecular characteristics, such as clonal type and the resistance elements they carried. In recent years it has become difficult to distinguish between CA- and HA-MRSA, especially with the significant rise in CA-MRSA cases (DeLeo et al. 2010; Falagas et al. 2013).

1.2.3 Transmission of MRSA

The increased incidence of CA-MRSA can be attributed to immunosuppression, community and environmental exposures; and high risk sexual behaviour such as having more than two sexual partners or having anonymous sex partners (Cole & Popovich 2013). The Centers for Disease Control and Prevention (CDC) include the following environmental factors as risks for MRSA transmission (<http://www.cdc.gov/niosh>):

- Crowding
- Frequent skin-to-skin contact
- Compromised skin integrity
- Contaminated items and surfaces
- Lack of cleanliness

In addition to these factors, it has been shown that patients with a high MRSA nasal burden (colonization) are more likely to develop infection (Datta & Huang 2008).

1.2.4 Diagnosis of MRSA infection

MRSA is diagnosed through standard laboratory procedures, which include phenotypic speciation and antibiotic resistance tests on either cultured clinical isolates, or performed directly on tissues, nasal swabs, blood cultures and other clinical specimens. Identification and susceptibility testing on isolates can be accomplished by a variety of manual and automated tests, including the VITEK®2 automated microbial identification and antibiotic susceptibility testing system (bioMérieux), API ID strip system (bioMérieux), disk diffusion tests (MAST Diagnostics) and E-tests (bioMérieux). Molecular tests like 16S rRNA polymerase chain reaction (PCR), the GeneOhm™ MRSA assay (BD) and the GeneXpert® MRSA assay (Cepheid) are available, but are less common, especially in resource-limited settings (Marlowe & Bankowski 2011). Molecular assays can be performed both on cultured isolates as well as certain clinical

samples. The potential advantage in performing them directly on clinical specimens is that this may allow for more timely initiation of appropriate antibiotic therapy and/or institution of infection control protocols. However, the evidence for the clinical or economic benefit of these rapid tests is still conflicting (Yang & Rothman 2004; Brown et al. 2005).

1.2.5 Treatment of MRSA infection

MRSA cannot be treated by conventional first-line therapy, such as oxacillin or similar penicillinase stable β -lactam antibiotics. Minor MRSA infections, such as boils or lesions, can be treated by incision and drainage of the affected area and decolonization of MRSA on the skin with antiseptic substances. In patients with systemic illness, trimethoprim-sulfamethoxazole, doxycycline, clindamycin, daptomycin, vancomycin and linezolid may be effective (Liu et al. 2011). While uncommon, there has been an increase in the reporting of Vancomycin intermediate *S. aureus* (VISA) and Vancomycin resistant *S. aureus* (VRSA) strains. This is a serious concern, since other last resort treatment options are limited and efficacy varies greatly depending on the clinical setting (Rivera & Boucher 2011).

1.2.6 Epidemiology

Both HA- and CA-MRSA pose a significant burden of high morbidity, mortality and cost of healthcare. Worldwide data shows that the highest rates (upwards of 50%) of MRSA are found in North America, South America and Asia. In contrast, most Scandinavian countries as well as the Netherlands have extremely low prevalence rates (Stefani et al. 2012). This is attributed to the search-and-destroy policies that have been implemented in the Netherlands and many Scandinavian countries. These policies vary in strictness, but aim to isolate and eradicate MRSA infections as soon as they are identified (Dekker & Broek 2010; Johnson 2011). The rates in Australia, China and Southern European countries range from 25 to 50% (Stefani et al.

2012). This is similar to data from most African countries that show that the prevalence of MRSA is lower than 50% (Falagas et al. 2013).

The first South African outbreak was recorded in 1986-1987 in Johannesburg (Falagas et al. 2013). Despite the implementation of improved infection control practices and the apparent decline of MRSA reported from 2006 to 2011, MRSA remains a concern (Falagas et al. 2013). At Tygerberg Hospital in 2008, 29% of all clinical specimens submitted to the laboratory were MRSA (Oosthuysen 2013). One study indicated a prevalence of 23% in Gauteng and 27% in KwaZulu-Natal (Moodley et al. 2010). A more recent study indicated that MRSA occurs in 29% to 46% of clinical *S. aureus* isolates in South Africa (Jansen van Rensburg et al. 2011).

To better understand the development of resistance and spread of MRSA in hospitals, it is important to study them on the genetic level. These studies allow researchers to distinguish between the spread of resistant isolates (clonal transmission) versus the spread of resistance genes (horizontal transmission) (Hanssen & Ericson Sollid 2006). It is vital to know the mechanism of the spread of MRSA within each community in order to properly monitor and control the progression of resistance.

Another concern is the increasing incidence of resistant strains amongst livestock, leading to livestock-associated MRSA (LA-MRSA). Livestock-associated MRSA infections are harder to treat and have been strongly associated with strains adept at immune evasion and carrying antibiotic resistance and other virulence factors. Infected meat also opens up another pathway for transmission of resistant strains into human hosts (Lindsay 2014).

1.3 SCCmec

1.3.1 Origin of the SCCmec element

Most resistance elements have a broad host range, however the large mobile genetic element, SCCmec, has so far only been found in staphylococci (Hiramatsu et al. 2001). This gene

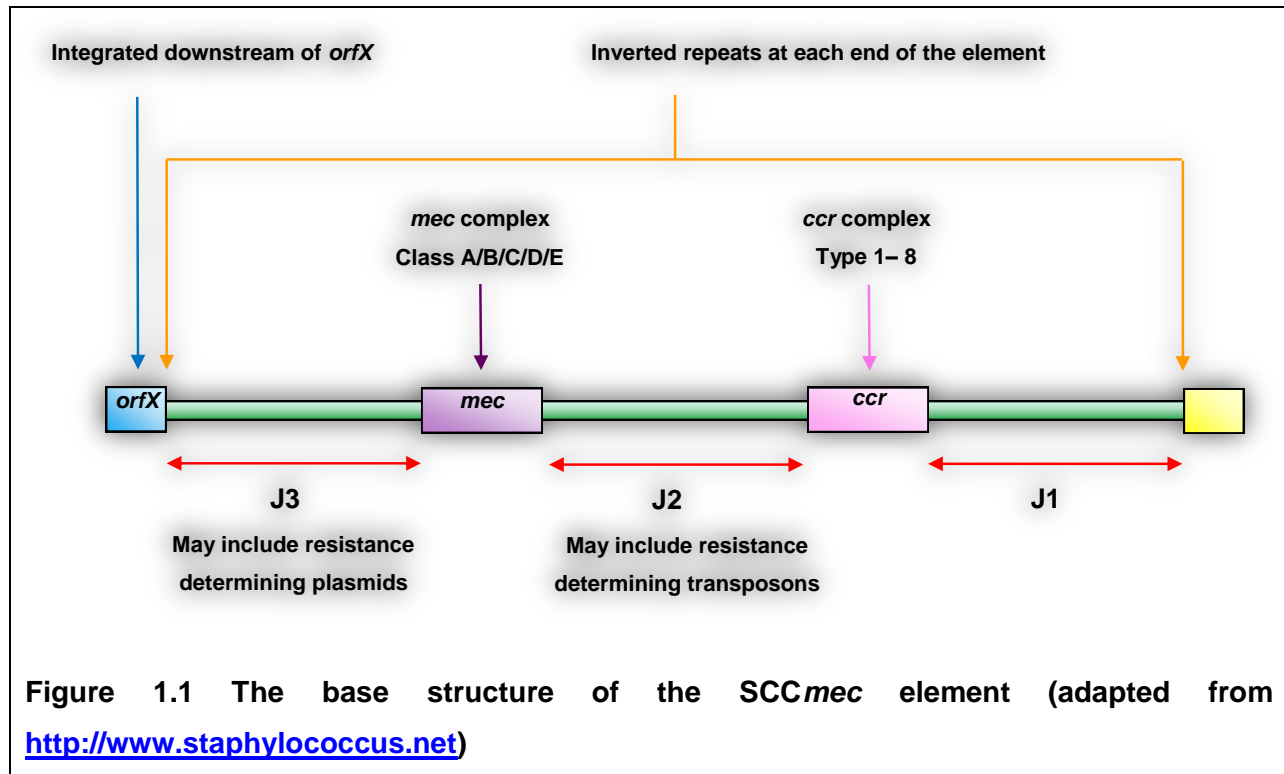
cassette is not inherent to *S. aureus* and the source was unknown (Pinho et al. 2001) until recently. Research now suggests that the SCC and *mecA* existed separately until *mecA* entered *S. aureus* through horizontal gene transfer between staphylococci and other Gram-positives shortly after the introduction of methicillin (Hanssen & Ericson Sollid 2006; Shore & Coleman 2013). It has been shown that SCC*mec* possibly originated from the acquisition of the *mecA* gene carried by *Staphylococcus fleuretti* (Tsubakishita et al. 2010). Another study suggested that *mecA* originating from *Staphylococcus sciuri* is a possible precursor for the *mec* genes found in most SCC*mec* elements today (Bouchami et al. 2011). Although it is generally accepted that MRSA developed from genes first found in coagulase negative staphylococci (CoNS), debate exists whether the reservoir for SCC*mec* elements is mainly CoNS or if it is MRSA itself. Researchers have found contradicting results and the topic warrants further investigation (Hanssen & Ericson Sollid 2006). The two contradicting theories of the origin of MRSA and SCC*mec* are explained by Deurenberg et al. (2007). The first is the single clone theory, which suggests that all MRSA clones have one common ancestor and that the dissemination of a couple of resilient clones drives the global spread of MRSA. The basis of this theory is that SCC*mec* was introduced into *S. aureus* only once. The second theory is widely accepted and suggests that MRSA evolved multiple times by independent lateral gene transfer events (Deurenberg et al. 2007; Stojanov et al. 2013). This is supported by the distribution of similar SCC*mec* elements in unrelated MRSA strains and different SCC*mec* elements in similar strains (Musser & Kapur 1992; Deurenberg et al. 2007; Stojanov et al. 2013).

1.3.2 Overall structure of SCC*mec*

The SCC*mec* element is a chromosomal mobile antibiotic resistance island. It is integrated in a site- and orientation specific manner near the *S. aureus* origin of replication, at the unique 15 bp bacterial chromosomal attachment site (*attB*SCC) in *orfX*. The function of *orfX* was unknown

until recently, when it was classified as a 23S rRNA methyltransferase (Shore et al. 2011).

Figure 1.1 shows the basic overall structure of the *SCC_{mec}* element.



The 21-67 kb *SCC_{mec}* element is a core component prone to genetic exchange between staphylococci (Hanssen & Ericson Sollid 2006). These elements are characterized by the presence of two main gene complexes, the *mec* complex (responsible for conferring resistance to methicillin through the *mecA* gene) and the chromosome cassette recombinase (*ccr*) gene complex, as well as various joining (J) regions (Ito et al. 2009). The core arrangement of the element is J3-*mec*-J2-*ccr*-J1; however these components can be arranged in various ways to form different *SCC_{mec}* types, making it an ideal tool for epidemiological purposes. Figure 1.2 shows the detailed structures of *SCC_{mec}* types I to VIII.

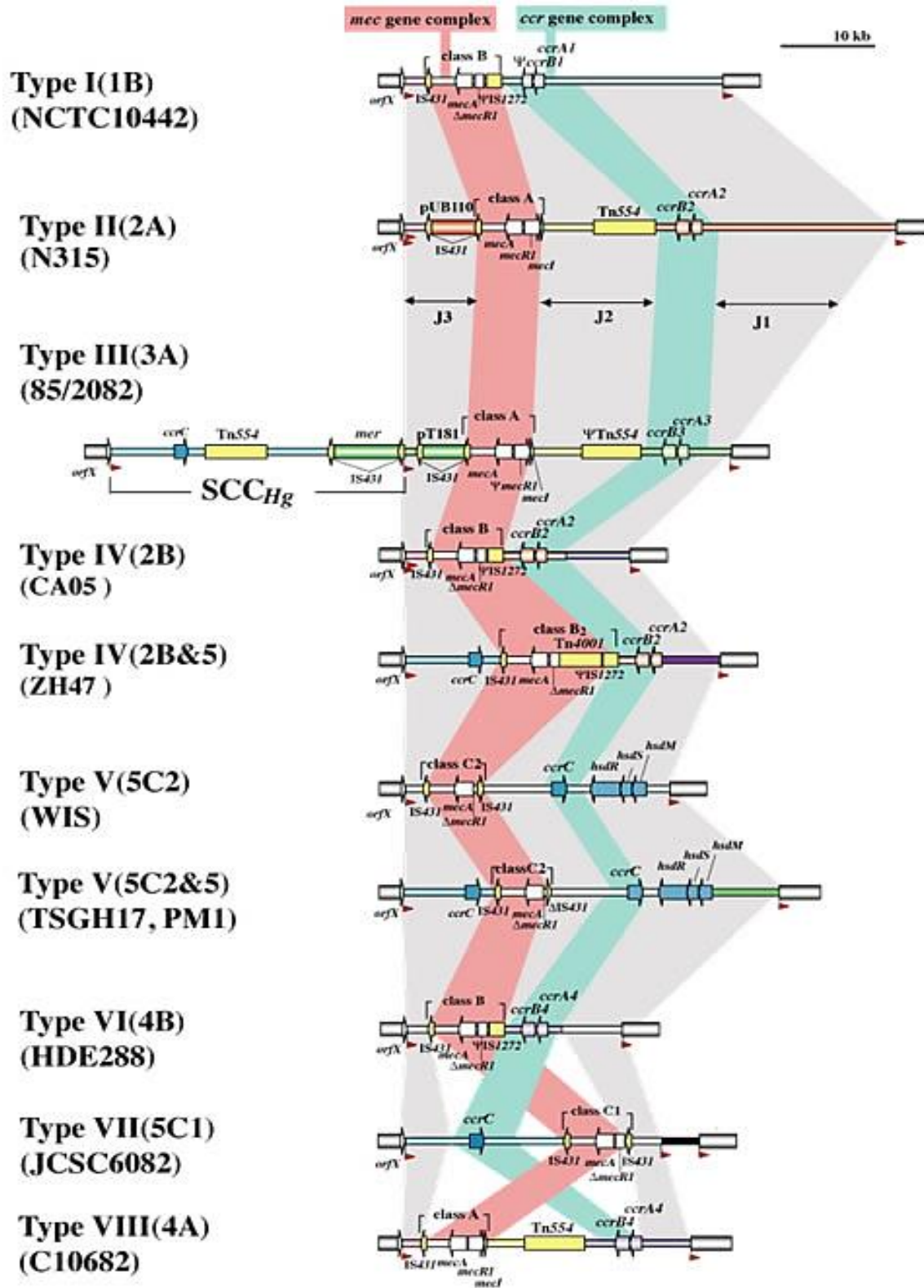


Figure 1.2 Specific SCCmec structures of types I to VIII (Source: Ito et al. 2009)

1.3.3 *ccr* complex

ccr encodes SCC*mec* specific serine recombinases of the invertase/resolvase family that recognize inverted repeats at the ends of the element allowing site- and orientation-specific integration and excision from staphylococcal genomes (Hiramatsu et al. 2001; Hanssen et al. 2004; Turlej et al. 2011). The *ccr* gene complex has four genes, *ccrA*, *ccrB*, *ccrC* and *ccrAA* and the functions of all but the last have been determined. *ccrAA* encodes an analogue of *ccrA* and usually occurs upstream from *ccrC*, but it has not been defined as a unique *ccr* gene complex (Monecke et al. 2011). Based on the combination of *ccrA*, *ccrB* and *ccrC*, two gene complexes can be formed: one with a combination of *ccrA* and *ccrB* allotypes, or another containing *ccrC* (Turlej et al. 2011). Different combinations of allotypes within these complexes have generated nine unique *ccr* types to date (Table 1.1). Types 1 through 5 have been identified in most staphylococci; however type 6 has only been seen in MR-CoNS isolates and type 7 and 8 are only found in MRSA isolates (Shore & Coleman 2013).

Table 1.1 Currently identified *ccr* gene complexes

<i>ccr</i> gene complexes	<i>ccr</i> genes	SCC <i>mec</i> types carrying the <i>ccr</i> gene complexes
Type 1	A1B1	I, IX
Type 2	A2B2	II, IV
Type 3	A3B3	III
Type 4	A4B4	VI, VIII
Type 5	C1	V, VII
Type 6	A5B3	-
Type 7	A1B6	X
Type 8	A1B3	XI
Type 9	C2	XII

Adapted from Ito et al. (2009) and (Wu et al. 2015)

1.3.4 *mec* complex

The *mec* gene complex carries the main resistance determining gene associated with MRSA, *mecA*, as well as two regulatory genes, *mecI* and *mecR1*. *mecI* encodes a transcription repressor protein and *mecR1* encodes a signal transduction protein. β -lactam antibiotics bind to the extracellular penicillin-binding domain of the MecR1 signal transducer which leads to autocatalytic cleavage in its cytoplasmic domain. MecR1 then functions as a protease by directly or indirectly cleaving any MecI repressor proteins that are bound to the operator region of *mecA* thereby activating transcription of the *mecA* gene (Hiramatsu et al. 2001).

In addition to *mecA*, *mecI*, and *mecR1*, one to four copies of insertion sequence (*IS431*) may be present in the *mec* complex. These elements are responsible for the MRSA characteristic multi-drug resistant phenotype, by clustering similar *IS* elements via homologous recombination (Hanssen & Ericson Sollid 2006). They also assist in the transfer of genes and plasmids into the chromosome (Hanssen & Ericson Sollid 2006). Table 1.2 shows the six currently identified *mec* gene complex classes and how the individual genes are arranged within each class.

Table 1.2 Currently identified *mec* gene complexes

<i>mec</i> gene complexes		SCC <i>mec</i> types carrying the <i>mec</i> gene complexes
class A	IS431- <i>mecA-mecR1-mecI</i>	II, III, VIII
class B	IS431- <i>mecA-ΔmecR1-IS1272</i>	I, IV, VI
class C1	IS431- <i>mecA-ΔmecR1-IS431</i> (IS arranged in the same direction)	VII,X
class C2	IS431- <i>mecA-ΔmecR1-IS431</i> (IS arranged in the opposite direction)	V, IX, XII
class D	IS431- <i>mecA-ΔmecR1</i>	
class E	<i>blaZ-mecALGA251-mecR1LGA251-mecILGA251</i>	XI

Adapted from (<http://www.staphylococcus.net>) and Hiramatsu et al. (2001).

1.3.5 Joining regions

Three distinct joining (J) regions are found within the SCC*mec* element, arranged in different ways depending on the SCC*mec* type. These regions were initially considered to be junkyard regions, but research showed that they may contain additional non-essential resistance determining elements, such as pseudogenes, plasmids and transposons (Zhang et al. 2012). Variations within J1 to J3 are used to assign SCC*mec* subtypes. Typically, the J1 region is found between the *ccr* complex and the end of the element, J2 is situated between *ccr* and *mec* and the J3 region is between the *mec* complex and *orfX* (Figure 1.1). Table 1.3 describes the additional resistance genes that may be found in the J regions of the SCC*mec* element. They are typically only found in the larger SCC*mec* types that typically circulate in HA-MRSA strains,

such as SCC*mec* types I, II and III (Turlej et al. 2011) possibly due to the fitness cost associated with acquisition of additional genes.

Table 1.3 Additional resistance elements that may be found in the SCC*mec* element

Element	Resistance	Region of SCC <i>mec</i>	SCC <i>mec</i> types carrying the element
pUB110	bleomycin; tobramycin	J3	IA, II, IVA
pT181	tetracycline	J3	III
Tn554	erythromycin; streptomycin	J2	II,VIII
ΨTn554	cadmium	J2	III
Tn4001	aminoglycosides	<i>mec</i> complex	IVc

Adapted from Turlej et al. (2011)

1.3.6 SCC*mec* types

At least twelve different SCC*mec* types, shown in Table 1.4, have been identified and described to date (Milheirico et al. 2007; Kondo et al. 2007; Ito et al. 2009; Lulitanond et al. 2013; Wu et al. 2015; <http://www.staphylococcus.net>). This is based on the combination of different *mec* and *ccr* gene complexes, of which numerous classes and allotypes have been identified. Several subtypes have also been described, based on variations within the J regions of the SCC*mec* element.

Table 1.4 The twelve currently published SCC*mec* types (as of August 2016)

SCC <i>mec</i> types	ccr gene complexes	<i>mec</i> gene complexes
I	1 (A1B1)	B
II	2 (A2B2)	A
III	3 (A3B3)	A
IV	2 (A2B2)	B
V	5 (C1)	C2
VI	4 (A4B4)	B
VII	5 (C1)	C1
VIII	4 (A4B4)	A
IX	1 (A1B1)	C2
X	7 (A1B6)	C1
XI	8 (A1B3)	E
XII	9 (C2)	C2

SCC*mec* types I, II and III were described in 2001 by Ito and colleagues (Ito et al. 2001) and are primarily associated with HA-MRSA (Grundmann et al. 2006). They are the largest of the SCC*mec* elements and carry many additional genes, including genes conferring resistance to erythromycin, spectinomycin and tetracycline. They also serve as a chromosomal deposit site for other resistance genes, and various integrated plasmids (Ito et al. 2001). The presence of these SCC*mec* elements is associated with a slower growth rate and a decrease in fitness in the absence of antibiotic pressure (Monecke et al. 2011). SCC*mec* III was initially reported as the largest element (Ito et al. 2001), but was later redefined as a composite of the SCC*mec* III region and another element called SCC*mercury*, encoding *ccrC*, a transposon Tn554 and the *mer* operon (Chongtrakool et al. 2006). After this discovery, multiple SCC*mec* III variants not

carrying the *SCCmercury* element were reported, suggesting that the two elements originated independently (Arakere et al. 2009). During this time, the name *SCCmercury* was changed to *SCC_{Hg}* to avoid confusion with *SCCmec* (Ito et al. 2009).

SCCmec type IV was the first element identified from CA-MRSA strains. It is smaller than *SCCmec* types I, II and III and commonly devoid of most other resistance genes (with the exception of *mecA*) (Ma et al. 2002). Recently, type IV has started circulating amongst healthcare facilities and is no longer a true CA-MRSA type. It is also the most diverse, most likely due to its small size, and has at least 8 reported subtypes (Ghaznavi-Rad et al. 2010).

The description of the small, community associated *SCCmec* type V isolated in Australia led to the discovery of the *ccrC* gene. Type V lacks any antibiotic resistance genes except for *mecA* and carries genes for a restriction-modification system that may aid in stabilization of the element on the chromosome (Ito et al. 2004). MRSA strains carrying *SCCmec* IV or V have also spread among livestock and are known as livestock associated MRSA (LA-MRSA) (Monecke et al. 2011). Interestingly, although they lack multidrug-resistance genes CA-MRSA strains are commonly associated with serious necrotizing pneumonia and SSTI due to the carriage of toxin genes encoding for Panton-Valentine leucocidin (PVL). Strains that are PVL positive are known to be extremely virulent and spread rapidly through communities (Monecke et al. 2011).

Strain HDE288, the model for the “pediatric” MRSA clone that originated in hospitals in Portugal, was found to contain *SCCmec* type VI. Type VI was originally misclassified as a variant of type IV, but further studies revealed that it had a different *ccr* allotype, *ccrAB4*, which led to its reclassification (Oliveira et al. 2006).

SCCmec type VII is suspected to be a community-acquired strain. It was detected in Japanese CA-MRSA isolates and described as a smaller element, similar in size to type IV and V and carrying a *mec* class C1, previously only found in *S. haemolyticus* (Berglund et al. 2008).

SCC*mec* type VIII was identified in a hospital associated Canadian epidemic MRSA isolate. Complete sequence data of this element revealed it was possibly generated through recombination with a *S. epidermidis* strain (Zhang et al. 2009).

In 2011, two novel SCC*mec* types, IX and X, were identified in LA-MRSA strains of the clonal complex 398. These strains were found primarily in pigs, as well as other livestock and individuals who had contact with them. This indicates that there is a true reservoir of MRSA strains circulating amongst livestock. SCC*mec* types IX and X were also found to harbour genes allowing detoxification of heavy metals (Li et al. 2011). SCC*mec* type XI was first sequenced by the Sanger Institute from the bovine MRSA strain LGA251 (sequence type 425) in England (Shore & Coleman 2013). This SCC*mec* type is very unusual due the presence of highly divergent *mecA*, *mecI*, *mecR1*, *blaZ*, and *ccr* genes. An arsenic resistance gene was also identified, which is particularly interesting as this gene has not previously been found in association with SCC*mec*, but has been seen in CoNS, MSSA, *Escherichia coli*, and *Pseudomonas fluorescens* (Shore et al. 2011).

Types IX and X share some of the features of type XI that may give clues to the origin of SCC*mec*. All three are considered to be from MRSA strains of animal origin and they carry genes encoding resistance to heavy metals. In addition, the divergent structure of SCC*mec* type XI suggests that it evolved in a different species or genus, and that it possibly contains ancestral forms of the gene complexes of SCC*mec* (Shore & Coleman 2013).

The most recent SCC*mec* type contained a novel *ccrC2* gene, which was found to be widely spread in CoNS isolates, but also seen in other staphylococcal isolates from China, France, Germany and the United States. This element was also flanked by a pseudo (Ψ) SCC element (SCC element lacking a functional *mec* or *ccr* gene) (Wu et al. 2015).

1.4 Molecular SCC*mec* typing methods

Being able to assign a SCC*mec* type correctly is an important requirement for epidemiological studies. SCC*mec* typing can also be utilized for the investigation into the origin and evolution of contemporary MRSA clones that have disseminated globally (Turlej et al. 2011). There are four approaches to molecular typing of the SCC*mec* element: restriction enzyme digestion, multiplex PCR (mPCR), real-time PCR and sequencing. Typing with the use of mPCRs is the most popular and economical of all four methods, however classification based on band sizes alone, may be misleading. Therefore sequencing the entire element remains the gold standard for typing, especially when characterizing a novel element. Unfortunately, sequencing is relatively expensive and the subsequent data analysis is complicated. As a result mPCRs are usually completed first to assess the need for sequencing. Below we summarize the existing mPCR approaches and how they have evolved.

Several mPCR based typing schemes have been designed for the identification of SCC*mec* elements in clinical *S. aureus* isolates and also for the sub-typing of some SCC*mec* types. These mPCRs are tools that type based on unique DNA band patterns observed by gel electrophoresis. The first known attempt was made by Oliveira and de Lencastre (2002). At the time of publication, only SCC*mec* types I to IV were described in the literature and the mPCR was designed to detect all of them as well as a few subtypes of I and III.

Shortly after this, a Canadian research team developed a new single assay to classify the types and subtypes I, II, III, IVa, IVb, IVc, IVd and V (Zhang et al. 2005). In addition to the first reaction, two other multiplex PCRs were designed to characterize the *mec* and *ccr* gene complexes; and a uniplex PCR to assign *ccr* 5. While it was more accurate and descriptive than the original mPCR designed by Oliveira *et al.* (2002) the multiple reactions necessary for full characterization were labor-intensive and therefore not suited for larger sample numbers.

Boye *et al.* (2007) developed a simpler mPCR using only four primer pairs that was able to distinguish between the five main *SCCmec* types described at the time. However, in light of the discovery of type VI and multiple subtypes of type IV, this method was rapidly considered outdated. In addition, Oliveira's group published an update to their mPCR strategy in 2007, which was able to identify and discriminate between types I to VI (Milheirico *et al.* 2007). This method is rapid and robust and it detects all the major HA-MRSA types that have spread across healthcare facilities and communities across the world. A mPCR very similar to the Milheirico method (Milheirico *et al.* 2007) was developed in 2010 that has the ability to better distinguish subtypes of type IV, however it shows no other significant advantage as a typing strategy (Ghaznavi-Rad *et al.* 2010).

A major breakthrough for *SCCmec* type assignment came with the design of six unique mPCRs by Kondo *et al.* (2007). Since this approach does not target specific *SCCmec* types, but rather specific loci, the first two mPCRs alone are potentially able to classify *SCCmec* types I to XI, with the exception of types VII and X. In most cases, this should be sufficient for epidemiological purposes (Turlej *et al.* 2011). When the last four mPCRs are added, a large variety of subtypes and the presence of additional resistance determining elements can be identified.

Chen *et al.* (2009) developed a multiplex real-time PCR for *SCCmec* typing using molecular beacons. The advantages of such an assay are the high sensitivity, reduced downstream analysis and lower risk of amplicon contamination. Up to 96 isolates can be typed within 4 hours. Unfortunately this assay was only designed to detect *SCCmec* types I - VI and VIII and is not suitable for subtyping. Molecular beacon probes are also more expensive in comparison to traditional PCR methods and are only cost-effective when used in bulk.

In summary, while many different PCR methods have been designed as strategies to type *SCCmec* elements, they do not perform equally or generate corresponding data (Ghaznavi-Rad *et al.* 2010). So far, the assay described by Kondo *et al.* (2007) is the most complete typing

scheme, however, since more than one mPCR reaction is required, it can become time consuming. To save time and streamline the typing process, a combination of the Milheirico (2007) and Kondo (2007) protocols may prove more effective. Initial screening done by the Milheirico (2007) protocol should be able to type the bulk of the isolates, since it types the main circulating strains seen worldwide. The Kondo (2007) protocol can then be reserved for cases where Milheirico (2007) is unable to assign a type, or unusual band patterns are observed.

If molecular typing by means of PCR reveals a potential novel *SCCmec* type or subtype, it is essential to determine the entire nucleotide sequence of the *SCCmec* element by sequencing. The *SCCmec* element must be analyzed to prove the presence of novel DNA sequences in, or combinations of the characteristic genes, pseudogenes and other noncoding regions (J regions). Secondly, mobile elements, mostly those that provide resistance to antimicrobials, such as plasmids, transposons or insertion sequences, must also be present in the J region. In addition, studying the genome may give insight into possible horizontal gene transfer (HGT) events, and how the *SCCmec* element continues to gain resistance to numerous antibiotics and heavy metals (Kuroda et al. 2001).

Owing to the ongoing confusion around *SCCmec* nomenclature, the International Working Group for *SCCmec* (IWG-SCC, <http://www.staphylococcus.net>) was formed to provide consensus guidelines for nomenclature and to establish minimum standards for identifying new *SCCmec* types. It is recommended that all new putative *SCCmec* types, along with accession numbers be submitted to the IWG for review and allocation of a name (Ito et al. 2009).

1.5 MR-CoNS

1.5.1 Clinical significance of MR-CoNS

Staphylococci are classified into two major groups, namely coagulase-positive staphylococci, represented almost exclusively by *S. aureus*; and coagulase-negative staphylococci (CoNS). All

staphylococci readily colonize the human skin and mucous membranes as opportunistic pathogens, but the ability of CoNS to cause infection is much less than that of *S. aureus* (Shore & Coleman 2013). However, the increasing number of vulnerable population groups, including the elderly, premature neonates and immunocompromised persons; is increasing the clinical significance that these organisms have (Becker et al. 2014). The ability of the most common member of the CoNS, *S. epidermidis*, to form biofilms is especially worrying in neonatal intensive care unit (NICU) settings (Becker et al. 2014). Furthermore, the levels of antibiotic resistance in hospital CoNS populations (specifically towards methicillin) are very high and can be upwards of 60% (Barbier et al. 2010). Further, CoNS commonly present with multidrug resistant phenotypes that dramatically increase morbidity in nosocomial infections (Widerström 2016).

Research done with clinical specimens suggest that MR-CoNS may act as a reservoir of *SCCmec* elements or their individual components (Bouchami et al. 2011). Although the mechanism for the transfer of *SCCmec* from MR-CoNS to methicillin-sensitive *S. aureus* (MSSA) is yet to be determined, the hypothesis is that horizontal gene transfer (HGT) occurs between commensal CoNS and MSSA leading to the evolution of MRSA (Hanssen & Ericson Sollid 2006; Barbier et al. 2010).

1.5.2 *SCCmec* types in MR-CoNS

Methicillin-resistant coagulase-negative staphylococci (MR-CoNS) have a more divergent *SCCmec* structure than other staphylococcal species, contain *ccr-mec* combinations that are not found in MRSA, and also have untypeable *ccr* allotypes (Ruppé et al. 2009). This diversity, along with a lack of a numbering system specific for CoNS hampers molecular typing of the *SCCmec* in species belonging to this group. *SCCmec* types II, III, IV and V which are found in MRSA are also very common in CoNS, however combinations of these types are abundant and no typing schemes have been developed to properly classify them (Zong et al. 2011). In

addition to the interesting SCC*mec* combinations, some elements may even lack one or both of the two major gene complexes, *ccr* and *mec*. Some *S. hom. hominis* and *S. haemolyticus* strains have been reported to have non-*mecA* SCC*mec* elements. These and other Ψ SCC elements such as the arginine catabolic mobile elements (ACMEs) are common amongst CoNS (Becker et al. 2014).

1.6 Problem statement

In South Africa, there is a lack of data concerning the molecular epidemiology of MRSA (Jansen van Rensburg et al. 2011). The majority of the existing data originates from the Gauteng and KwaZulu-Natal provinces (Moodley et al. 2010) and is outdated. Recent studies done at Tygerberg Academic Hospital, Cape Town, suggest a high prevalence of multidrug resistant MRSA (Oosthuysen 2013), which demonstrates that further studies addressing the molecular epidemiology of MRSA are necessary in our setting. In addition, preliminary data generated in our laboratory suggests that the largest cluster of our MRSA isolates from blood culture specimens (34%) may contain a novel SCC*mec* type (Karayem 2014). Furthermore, this study also identified a potentially novel variant of SCC*mec* type I in 17% of the isolates. Initial typing using multiplex PCR (Milheirico et al. 2007) suggests that the novel variant consists of *ccrC* and *ccrA1B1* with a class B *mec* gene. This novel SCC*mec* type was also identified in a *S. capitis* isolate, suggesting that this novel type may have evolved from CoNS. We believe that the novel SCC*mec* may be a combination of a Class A *mec* and *ccrC* (Type 5A). These novel and novel variant SCC*mec* types therefore warrant further characterization.

Aims and Objectives

We aimed to describe the epidemiology and genotypic characteristics of MRSA and CoNS strains isolated at Tygerberg Hospital, South Africa, with a specific focus on determining the prevalence, source and full genetic characterization of a potentially novel *SCCmec* type.

We hypothesized that this *SCCmec* type was indeed novel and resulted from horizontal gene transfer from CoNS strains.

Aims

1. To fully characterize the novel and novel variant *SCCmec* elements.
2. To describe the epidemiology and *SCCmec* types present in methicillin-resistant CoNS strains isolated at Tygerberg Hospital, South Africa.

Objectives

1. To provisionally characterize the novel and novel variant elements and confirm the presence of generic regions using multiplex PCR assays.
2. Based on the results of Objective 1,
 - a. Review the *SCCmec* typing results of isolates stored during the course of previous studies to determine whether the novel or novel variant *SCCmec* types were present in older isolates.
 - b. Perform *SCCmec* typing on a collection of MR-CoNS isolates in order to determine whether the novel and/or novel variant *SCCmec* types were present in local CoNS isolates.
 - c. Perform next-generation sequencing (NGS) analysis on a selected subset of isolates to characterize the structure of the novel and novel variant SCC elements.

Chapter 2: The SCC*mec* typing of MRSA and MR-CoNS isolates according to the method of Milheirico et al. 2007

2.1 Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is an increasing problem in hospitals and causes an increase in morbidity and mortality associated with staphylococcal infections. Methicillin resistant coagulase negative staphylococci (MR-CoNS) are also commonly isolated in hospital settings, and epidemiological studies show that methicillin resistance rates in healthcare settings were higher than 70% (Diekema et al. 1999; Decousser et al. 2015). The clinical impact of high resistance rates in CoNS remains unclear, because it is difficult to distinguish between contamination and true infection in CoNS cases (Tashiro et al. 2015).

The core MGE responsible for methicillin resistance in staphylococcal species is the SCC*mec* element. In MRSA these elements have characteristic structures that can be used for epidemiological typing. Along with MLST (multi locus sequence typing), *spa* typing and PFGE (pulsed-field gel electrophoresis), SCC*mec* typing is an essential tool in the identification of the diversity and mechanism of transmission of MRSA clones amongst populations. Currently, there are 12 SCC*mec* types and multiple subtypes described in the literature. Many SCC*mec* typing schemes exist, but most are laborious and time-consuming to perform. The multiplex PCR (mPCR) assay described by Milheirico and colleagues (Milheirico et al. 2007) is a relatively fast way of screening MRSA isolates for the most common SCC*mec* types (I to VI).

The purpose of this study was to investigate putative novel and novel variant SCC*mec* types previously found in our setting by performing molecular typing using the Milheirico (2007) protocol. We also screened prospectively collected MR-CoNS isolates to determine whether this population harboured the novel elements as suggested by the discovery of the novel type in a *S. capitis* isolate.

2.2 Methods

2.2.1. Sample selection

Staphylococcus aureus We planned to investigate 90 MRSA isolates (from blood cultures and nasal swabs) that had been stored from 2010 to 2011 as part of the previous study which first identified the putative novel and novel variant SCCmec types (Karayem 2014). Of the 90, 87 were viable and were included in this study.

Coagulase negative staphylococci

Four hundred seventy CoNS isolates from blood cultures processed by the National Health Laboratory Service (NHLS) microbiology laboratory, Tygerberg, were prospectively collected and stored. These blood cultures were taken as part of routine patient care. Isolates were identified and susceptibility profiles determined by the routine Standard Operating Procedures (SOPs) of the NHLS. This included Gram staining, catalase, DNase (DNA hydrolysis) tests, Pastorex Staph Plus (Bio-Rad, South Africa) rapid agglutination test and culture on MSA (mannitol salt agar) to distinguish between *S. aureus*, CoNS and other Gram positive organisms. The majority of CoNS isolates were not considered to be clinically significant (i.e. most likely skin contaminants) and therefore species identification was not done. Of the 470 collected isolates, 170 randomly selected isolates were screened for methicillin resistance and total of 100 methicillin resistant isolates were identified..

Selected patient data on all samples were collected including age and gender of the patient and from which ward the sample was sent. This study was approved by the HREC (Health Research Ethics Committee) of Stellenbosch University with the approval number S15/03/065.

2.2.2. Sample storage

Microbank Tubes (Pro-Lab Diagnostics) were used for sample storage. Each tube contains 20 to 30 sterile beads along with a cryopreservative, which allows long term storage of isolates at -70 to -80°C. A loop full of colonies from pure overnight cultures (MSA plates, NHLS Media Laboratory, South Africa) was inoculated on solid horse blood agar (NHLS Media Laboratory, South Africa). The tubes were then inverted 4 to 5 times to emulsify the organisms and incubated at room temperature (25°C) for 2 minutes. After incubation a pipette with a sterile, disposable tip was used to remove as much of the cryopreservative as possible. The tubes were then stored at -70 to -80 °C.

2.2.3. Cefoxitin screening of CoNS isolates

Stored CoNS samples were phenotypically screened for methicillin resistance to select a total of 100 MR-CoNS isolates for molecular *SCC_{mec}* typing. Following the Clinical and Laboratory Standards Institute standards (CLSI 2013), the cefoxitin (FOX) disk diffusion test was used instead of oxacillin. FOX is a stronger inducer of the *mecA* gene than oxacillin and therefore produces more accurate results.

Selected isolates were subcultured onto blood agar from single Microbank beads and incubated at 37° C for 18 to 24 hours. Four to five colonies were re-suspended in up to 5 mL of saline to produce a McFarland standard of 0.5. A sterile swab was used to inoculate the suspension onto the surface of Mueller-Hinton agar plates (NHLS Media Laboratory, South Africa) and once the plates had dried, 30 µg FOX disks (MAST Diagnostics, UK) were placed in the centre of each plate using a sterile needle. The plates were incubated aerobically for 24 hours at 37°C, after which the inhibition zone diameters were measured. The disk diffusion results were interpreted using published CLSI criteria to determine FOX resistance in CoNS species, where an inhibition zone of ≥ 25 mm is considered to be sensitive and ≤ 24 mm is resistant (CLSI 2013).

2.2.4. Speciation of MR-CoNS isolates

To gain a better idea of the range of different species in our MR-CoNS population, the MR-CoNS isolates were speciated using the VITEK®2 system (bioMérieux, South Africa). This is an automated biochemical identification platform which uses Advanced Colorimetry™ to detect the results of multiple biochemical tests on specific identification (ID) or antimicrobial susceptibility test (AST) cards. The assay was done according to the NHLS standard operation procedure (SOP) for the VITEK®2. Individual test results were indicated as “+” (positive), “-” (negative) or “?” (weak reaction) and analysed by the software to give an organism ID within 4 to 6 hours.

2.2.5. DNA extraction of MRSA and MR-CoNS isolates

A crude boil method was implemented for DNA extraction of stored organisms. A Microbank bead was plated onto a blood agar plate and incubated at 37 °C overnight. After incubation, plates were inspected and four to five colonies were selected and re-suspended in 200 µL of nuclease free water in 1.5 mL microcentrifuge tubes. Nuclease free water was used as a cost-effective alternative for Tris-EDTA (TE) buffer, since it was observed that the DNA remained stable during storage at -20°C. The tubes were incubated at 95°C for 30 minutes, followed by incubation at -80°C for another 30 minutes. Tubes were allowed to thaw at room temperature (25°C) and then centrifuged for 10 minutes at 14000 x *g*. The supernatant containing the DNA was aliquoted to avoid freeze-thaw induced DNA degradation and stored at -20° C until further use. Samples were spun down for 3 minutes at 14000 x *g* before each PCR to pellet any cell debris.

2.2.6. Milheirico mPCR on MRSA and MR-CoNS isolates

SCC*mec* typing was performed on all stored MRSA isolates using the multiplex PCR (mPCR) method previously described by Milheirico et al. (2007). This was done to confirm previous SCC*mec* typing performed on these isolates (Karayem 2014). The mPCR used 20 different

primers (Integrated DNA Technologies, South Africa) (listed in Table 2.1) to amplify specific targets within the SCC*mec* element. These included sequences in the *mec* gene and *ccr* gene complex, and the joining regions J1 and J3 and an internal control target, *mecA* (162 bp), to validate each reaction and to confirm methicillin resistance. The combination of these targets was used to classify isolates into SCC*mec* types by comparing their band patterns to those of reference strains (Table 2.2) as shown previously (Milheirico et al. 2007). A no template control (NTC) with nuclease free water was added to each PCR run to determine possible contamination.

Briefly, each reaction contained 1 µL of template DNA, 1X KAPA 2G Fast Multiplex Mix (KAPA Biosystems, South Africa), the ten sets of primer pairs with concentrations shown in Table 2.1 and nuclease-free water to bring to volume (25 µL).

Amplification consisted of an initial denaturing step at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 45 seconds. A final extension step at 72°C for 3 minutes concluded the PCR. Samples were held at 4°C until further use.

Amplification products (for expected sizes, please refer to Table 2.1) were visualized on 3% SeaKem LE Agarose (Lonza, South Africa) gels in tris-acetic acid-EDTA (TAE) buffer. The KAPA Universal DNA Ladder (KAPA Biosystems, South Africa) was used for band size reference (Addendum A: Materials). Gels were loaded with a mixture of 10 µL of sample/ladder and 2 µL of NovelJuice (GeneDireX, South Africa) loading dye, a non-toxic alternative to ethidium bromide staining (Addendum A: Materials). The gels ran for 70 to 80 minutes at 10 - 12 V/cm using the PowerPac Basic power supply system (Bio-Rad, South Africa) and amplicon bands were visualised with the UVIttec Alliance 2.7 gel documentation system (UVIttec, UK) and compared to the six reference SCC*mec* strains (Table 2.2).

Table 2.1 List of primers used for the Milheirico (2007) protocol

Name	Sequence (5' to 3')	Target: Type; <i>region</i>	Size (bp)	Concen- tration
kdpF1	AATCATCTGCCATTGGTGATGC	II; <i>J1</i>	284	0.2 µM
kdpR1	CGAATGAAGTGAAAGAAAGTGG			0.2 µM
CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	I; <i>J1</i>	495	0.4 µM
CIF2 R2	ATTTACCACAAGGACTACCAGC			0.4 µM
RIF5 F10	TTCTTAAGTACACGCTGAATCG	III; <i>J3</i>	414	0.4 µM
RIF5 R13	GTCACAGTAATTCCATCAATGC			0.4 µM
SCCmec III J1 F	CATTTGTGAAACACAGTACG	III; <i>J1</i>	243	0.4 µM
SCCmec III J1 R	GTTATTGAGACTCCTAAAGC			0.4 µM
SCCmec V J1 F	TTCTCCATTCTTGTTTCATCC	V; <i>J1</i>	377	0.4 µM
SCCmec V J1 R	AGAGACTACTGACTTAAGTGG			0.4 µM
mecI P2	ATCAAGACTTGCATTTCAGGC	II, III; <i>mec</i>	209	0.8 µM
mecI P3	GCGGTTTCAATTCACTTGTC			0.8 µM
dcs F2	CATCCTATGATAGCTTGGTC	I, II, IV, VI; <i>J3</i>	342	0.8 µM
dcs R1	CTAAATCATAGCCATGACCG			0.8 µM
mecA P4	TCCAGATTACAACCTTCACCAGG	Internal control	162	0.8 µM
mecA P7	CCACTTCATATCTTGTAACG			0.8 µM
ccrB2 F2	AGTTTCTCAGAATTCGAACG	II, IV; <i>ccr</i>	311	0.8 µM
ccrB2 R2	CCGATATAGAAWGGGTTAGC			0.8 µM
ccrC F2	GTA CTGTTACAATGTTTGG	V; <i>ccr</i>	449	0.8 µM
ccrC R2	ATAATGGCTTCATGCTTACC			0.8 µM

Table 2.2 Reference strains used in the Milheirico (2007) protocol

SCC<i>mec</i> type	Reference Strain	Origin
I	BAA-38	ATCC (American Type Culture Collection)
II	BAA-1681	ATCC
III	BAA-39	ATCC
IV	BAA-1680	ATCC
V	WIS	Division of Medical Microbiology, University of Cape Town
V	BAA-1688	ATCC
VI	BAA-42	ATCC

2.3 Results

2.3.1. Cefoxitin screening of CoNS isolates

One hundred seventy of the 470 CoNS isolates collected were screened for methicillin resistance using the cefoxitin (FOX) disk diffusion test. 40.6% (69/170) were FOX sensitive, 58.8% (100/170) were FOX resistant and a single isolate was deemed non-viable after repeated attempts to culture from Microbank beads. The average inhibition zone was 31 mm for the sensitive isolates and 16 mm for the resistant isolates. An example of a resistant isolate is shown in Figure 2.1.

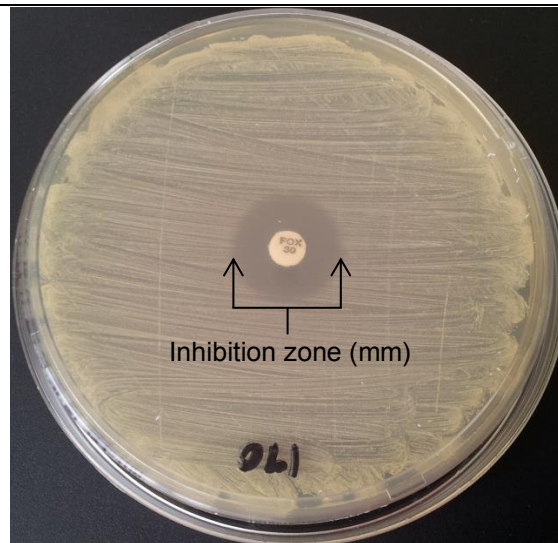
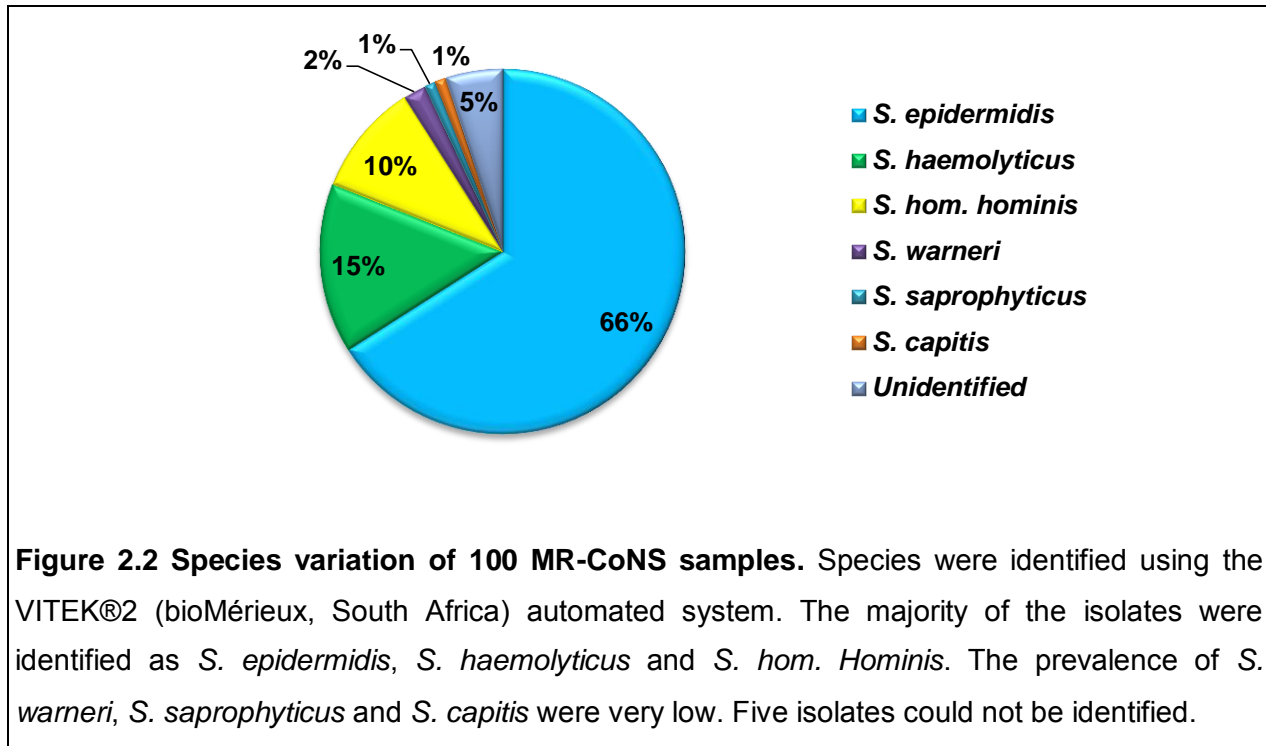


Figure 2.1 A MR-CoNS isolate as determined by the cefoxitin disk diffusion test. The arrow shows how the zone of inhibition zone was measured.

2.3.2. VITEK®2 speciation of MR-CoNS isolates

A total of 100 MR-CoNS samples were collected of which 19 were previously identified (18 *S. epidermidis*, 1 *S. capitis*). We identified the remaining 81 isolates with the VITEK®2 (bioMérieux, South Africa) automated analyser. As shown in Figure 2.2, methicillin resistance in our isolates is spread across a wide range of CoNS species commonly found in humans, of

which *S. epidermidis*, *S. haemolyticus* and *S. hom. hominis* form the majority. The VITEK®2 (bioMérieux, South Africa) was not able to discriminate between two or more species and identify 5% of the isolates.



2.3.3. Milheirico mPCR (MRSA isolates)

We performed molecular typing of 87 viable retrospective MRSA isolates, using the Milheirico (2007) protocol. The SCC_{mec} typing results are summarised in Figure 2.3.

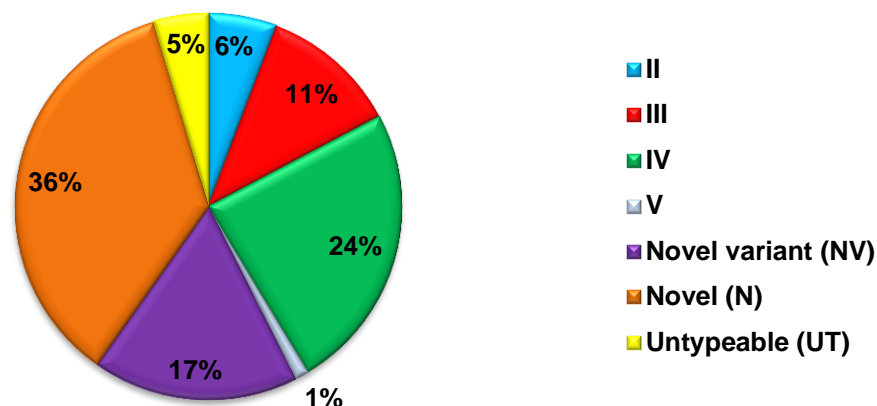


Figure 2.3 SCCmec typing. The results of molecular SCCmec typing on 87 MRSA isolates are shown. The majority of the isolates belonged to the N (orange), type IV (green) and NV (purple) groups. Types III and II (red and blue respectively) had lower prevalence and only one type V isolate was found. No SCCmec type I or VI isolates were identified. 5% of the isolates were untypeable by current methods (yellow).

The putative novel and novel variant SCCmec elements in our MRSA isolates represent 36% and 17% of the population respectively. The rest of the population comprised of SCCmec types IV (24%), III (11%) and II (6%).

Initially, we used strain BAA-1688 as a reference and control for SCCmec type V (Table 2.2), however after close inspection of the band patterns, we determined that this strain does not amplify the 377 bp SCCmec V J1 target. We performed a uniplex PCR for this target in BAA-1688, which was unsuccessful (results not shown). The Division of Medical Microbiology, University of Cape Town (C Moodley) kindly supplied us with an alternative SCCmec type V strain, WIS, and we compared the two strains (Figure 2.4) showing successful amplification of the J1 target (377 bp) in WIS.

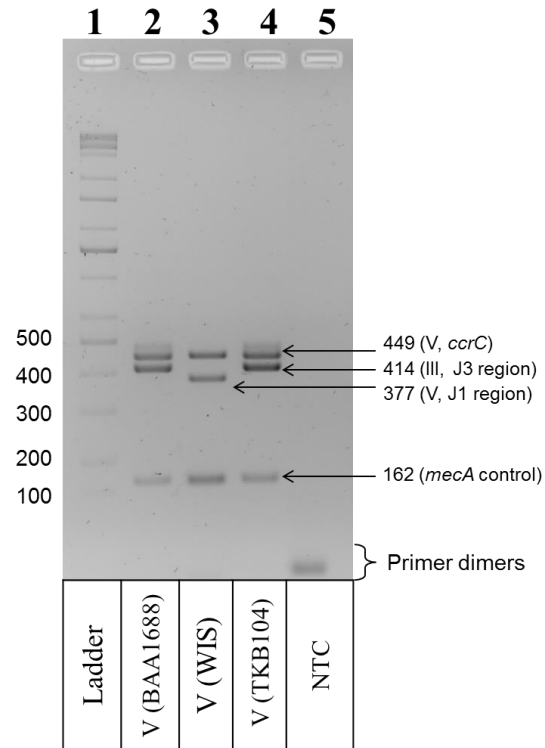
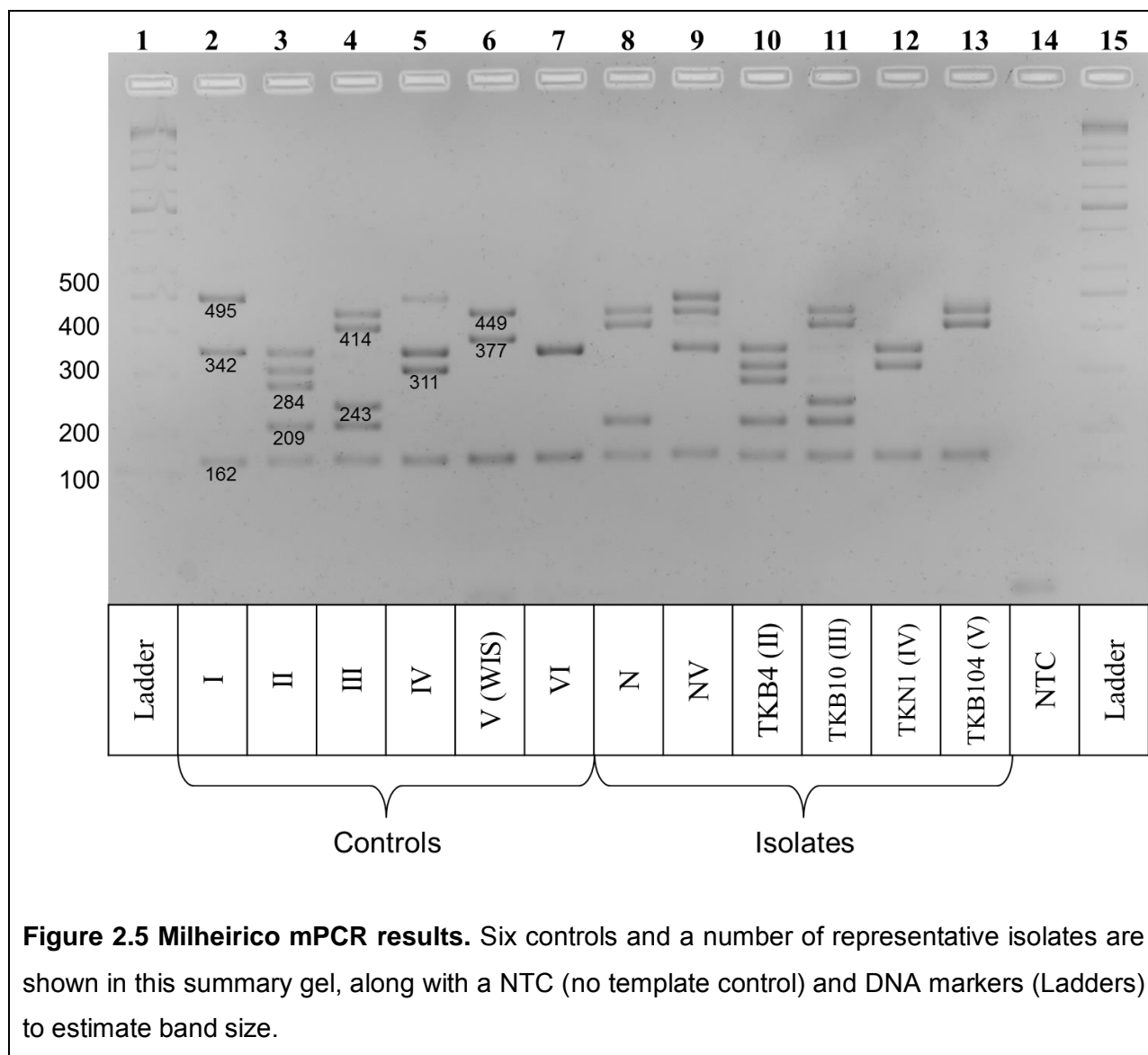


Figure 2.4 Milheirico mPCR. A comparison between two *SCCmec* type V reference strains, BAA-1688 and WIS, and a Type V variant isolate from our study. A DNA marker (ladder) and NTC were included.

BAA-1688 is possibly a type V variant missing the J1 region, as is the type V isolate that we typed, but further investigation will be necessary to confirm this. We continued the rest of the study using strain WIS as the control strain for *SCCmec* type V.

Figure 2.5 shows a typical *SCCmec* typing result for the Milheirico (2007) protocol.



Lanes 2 – 7 show the six controls that were included in each run for band pattern comparison. The type III control also contains the 449 bp product, *ccrC*, which is an indication that it may carry the SCC_{Hg} element. Lane 8 shows the novel type (N), which had a similar pattern to type III, with the absence of the J1 band (243 bp). The novel variant (NV, Lane 9) had a band pattern identical to type I with the addition of a product resembling *ccrC* (449 bp). One type V isolate was identified and it shared the band pattern of the type V control, BAA-1688 (Figure 2.4). Type

I and Type VI were not observed. Of the 87 isolates, 5% were untypeable and a single isolate had a failed PCR reaction.

2.3.4. Milheirico mPCR (MR-CoNS isolates)

In addition to the molecular typing of our MRSA isolates, we also typed all 100 of our MR-CoNS isolates. A typical mPCR result for MR-CoNS isolates is shown in Figure 2.6.

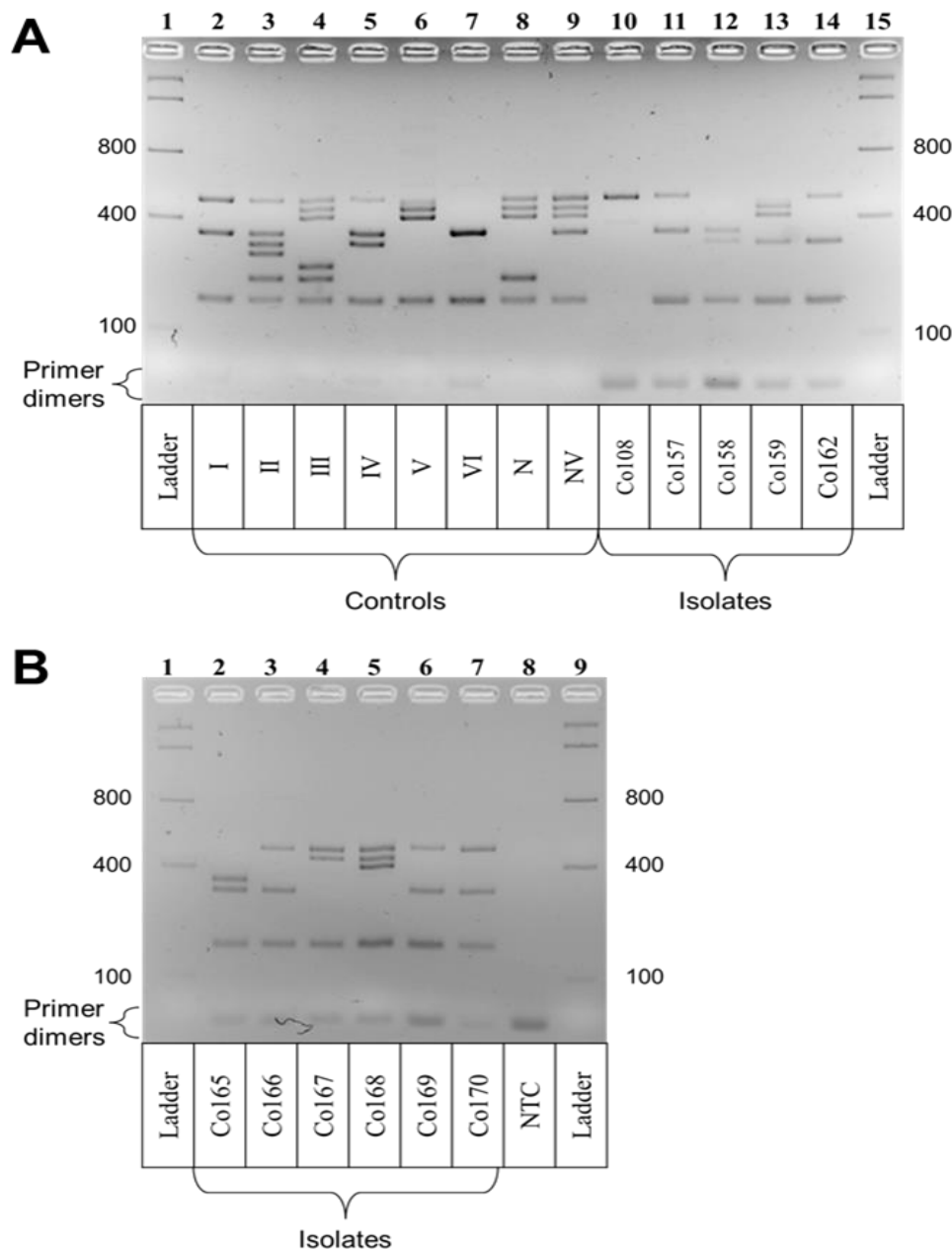


Figure 2.6 Milheirico mPCR on MR-CoNS isolates: A. Six controls and five isolates are shown, including DNA markers (Ladders); B. Additional isolates from the same PCR are shown, along with DNA markers (Ladders) and an NTC (no template control).

Some non-specific binding was observed around the largest targets for the controls of *SCCmec* type III, V and the novel and novel variant types. However, this did not impact on typing, since

each type still showed a unique band pattern. The CoNS isolates showed a more diverse set of *SCCmec* elements, and the majority (57%) could not be assigned by the standard MRSA *SCCmec* typing scheme. A largest typeable subset of the MR-CoNS isolates were type IV (35%), followed by type I (4%) and type V (3%, similar to the variant BAA-1688). Only one isolate carried the novel type. As shown in Figure 2.6 A (Lane 14) and B (Lanes 3, 6, 7), another *SCCmec* type I-variant was found in our CoNS population that differs from the novel variant found in the MRSA group, as it no longer has amplification in the J3 region as seen in type I, but has a band corresponding to *ccrB2*, which is present in types II and IV, and does not seem to carry the *ccrC* gene found in the MRSA novel variant. No novel variant band patterns were observed in any of the MR-CoNS isolates and only a single isolate was found with the novel band pattern identified in the MRSA group. Two *SCCmec* type V variants were found that were similar in band pattern to BAA-1688. A small number of CoNS isolates (11%) only had amplification of the internal control (*mecA*) and three non-*mecA* isolates (with amplification of other targets) were identified.

2.4 Discussion

2.4.1. MR-CoNS screening and speciation

The levels of methicillin resistance in our CoNS population (58.8%) were much lower than expected, since CoNS are often reported to have higher methicillin resistance rates than *S. aureus*, with levels ranging from 75% to as high as 90% (Diekema et al. 1999; Garza-González et al. 2010; Decousser et al. 2015). Mert et al. (2011) found that methicillin resistance levels in CoNS isolated from blood cultures is lower in true bacteraemia compared to contaminants. CoNS identified from blood culture are only considered to be significant if they are isolated from two or more consecutive blood cultures within a 24 hour period (Bekeris et al. 2005). However, our set of isolates effectively represents a convenience sample, and we do not know whether the CoNS in our sample were clinically significant or not, since the clinical data were not available for this study. A more stringent isolate and data collection strategy will allow the researcher to differentiate between true pathogens and contaminants and therefore give a more accurate clinical picture of methicillin resistance in CoNS populations. These strategies require intensive data capturing of blood cultures to determine the time samples were collected or registered in order to ensure the criteria for clinical significance is met. Furthermore, the clinical findings of physicians may be compared to the blood culture results.

The three most commonly isolated CoNS species from humans, *S. epidermidis*, *S. haemolyticus* and *S. hom. hominis* formed the majority of our MR-CoNS population. A small percentage of the isolates had identification results with low discrimination between two or more species which can be an indication of mixed cultures. Further analysis via a β -Glucosidase (bGLU) test is necessary to determine the species. Mixed cultures are a common problem during CoNS collection and storage, since it is challenging to differentiate between CoNS species directly from culture plates. They can become confounding in downstream reactions, especially if

culturing is done multiple times for different experimental procedures. To avoid this, it is essential to subculture from a single colony before storage on Microbank beads.

2.4.2. Multiplex PCR troubleshooting

Multiplex PCRs (mPCRs) such as the Milheirico (2007) protocol are useful epidemiological tools, since they can identify multiple targets from a single organism or range of different organisms. The majority of *SCCmec* typing schemes rely on the use of mPCRs. However, the nature of these mPCRs can make it difficult to optimize and to interpret results, as they produce multiple targets that are often similar in size and are therefore easily confused. It is essential to find the correct balance of PCR reaction components and thermocycling conditions to produce the selected PCR targets. One of the biggest problems of mPCRs is finding the ideal annealing temperature. Using an annealing temperature of 5 degrees below the primer's melting temperature (T_m) is usually sufficient for uniplex PCRs, but the presence of multiple primers may cause complications. If the annealing temperature is too low, non-specific bands may appear, and higher temperatures can cause some target regions to fail during amplification. Troubleshooting includes running the PCR at a range of different annealing temperatures to find the best result. In addition, the concentration of primers and $MgCl_2$ can be adjusted to optimize the PCR reaction once the ideal annealing temperature has been selected.

Agarose gel electrophoresis conditions can also adversely affect the interpretation of results if they are not set up correctly. The gel concentration, runtime and voltage need to be selected according to the number and size of the expected targets. Visual inspection of gel pictures at different time points will assist in the selection of the ideal runtime. Targets that range from 200 bp to 1000 bp in size can be run on 2% agarose gels. Targets smaller than 200 bp will have better separation on more concentrated gels (3%) and targets larger than 1000 bp will separate better on less concentrated gels (1.5%). The targets in the Milheirico (2007) protocol only range from 160 bp to 500 bp in size, however the high number of targets and similarities in target sizes

require a 3% gel for separation of targets to be acceptable for interpretation. Running gels for short periods of time can cause misinterpretation of targets and consequently incorrect type assignment. Software is available for cases where manual analysis may lead to error, for example extremely small base pair differences (<10 bp) between targets.

2.4.3. Milheirico mPCR on MRSA and MR-CoNS isolates

The advantage of using the Milheirico (2007) protocol for *SCCmec* typing is the short time-to-completion, which is approximately four hours, including DNA extraction. However, if samples are not batched, it can become laborious and expensive since each run requires the addition of six *SCCmec* controls. The protocol may therefore be more useful in settings where an existing collection of isolates will be screened, rather than ad hoc screening. Questions may be raised over the relevance of this protocol, since there are already 12 *SCCmec* types described in the literature, and this assay is only designed to assign *SCCmec* types I to VI. However, *SCCmec* types I to V are the types found in healthcare facilities globally, whereas types VI to XII are either local or livestock-associated types that are not connected to globally spread clones.

In our setting, the Milheirico (2007) protocol proved to be an ideal screening tool that identified both medically relevant *SCCmec* types and revealed possible novel and novel variant types. Isolates that cannot be typed with this protocol, can be investigated further using more comprehensive typing schemes, such as the one developed by Kondo et al. (2007).

While the Milheirico (2007) protocol is an effective screening tool for *SCCmec* types in MRSA isolates; its utility for MR-CoNS species is limited by their large diversity and the divergent structures of their *SCCmec* types. This highlights the need for a structured *SCCmec* typing scheme for CoNS species (Zong et al. 2011). The diversity and high rates of resistance in MR-CoNS suggest that they may be a reservoir for *SCCmec* types and facilitate the formation of new *SCCmec* types (Hanssen & Ericson Sollid 2007). The suggested mechanism for the

formation of new *SCCmec* types is horizontal gene transfer (HGT) between staphylococcal species (Hanssen & Ericson Sollid 2007; Garza-González et al. 2010; Mert et al. 2011; Zong et al. 2011). This does not appear to be the case for the origin of the novel and novel variant *SCCmec* types identified in our MRSA population. The novel and novel variant were similar to *SCCmec* types III and I respectively and together with *SCCmec* type III they formed the majority of our MRSA population. Conversely, screening of the MR-CoNS population did not identify the novel variant *SCCmec* element or *SCCmec* type III, and only a single novel *SCCmec* element. This suggests that the novel elements may have evolved by transmission and/or recombination events within the MRSA population and not through HGT between species.

In our study, the majority of MR-CoNS isolates were untypeable by the conventional *SCCmec* typing schemes and mainly carried combinations of published types or had uncharacterized band patterns. A small number of CoNS isolates (11%) only had amplification of the internal control (*mecA*), which may be an indication of truncated *SCCmec* elements or sequence changes in the *ccr* complex, which is known to be the most diverse in MR-CoNS isolates (Zong et al. 2011). Three non-*mecA* isolates (with amplification of other targets) were also identified, indicating that an alternative mechanism of methicillin resistance may be present in these isolates.

Among the typeable isolates, the most common *SCCmec* type found in our MR-CoNS population was *SCCmec* type IV. This is consistent with other published MR-CoNS *SCCmec* typing data (Jamaluddin et al. 2008; Barbier et al. 2010; Garza-González et al. 2010). The persistence and dissemination of *SCCmec* type IV and other smaller elements across both MRSA and MR-CoNS populations may be linked to increased mobility and its smaller size (Strandén et al. 2009; Barbier et al. 2010; Mert et al. 2011).

2.4.4. Conclusion

Preliminary *SCCmec* typing revealed that the largest cluster of MRSA isolates in our setting belonged to putative novel and novel variant *SCCmec* types. We determined that they were closely related to *SCCmec* types III and I, which are associated with HA-MRSA strains. The Milheirico (2007) protocol was found to be a suitable screening method; however additional typing methods are necessary to effectively characterize the novel and novel variant *SCCmec* types.

The MR-CoNS population was initially considered to be a reservoir for the novel and novel variant SCC elements; however our results showed that the novel types were absent in the MR-CoNS population. A limitation of this study is that the isolate collection for the MRSA and CoNS groups was done at different time points (2010 and 2014, respectively). There is a possibility that the novel elements were initially present in the MR-CoNS population, but did not convey any evolutionary advantage to the MR-CoNS population, and were therefore lost. It may also be useful to investigate whether the novel elements are still present in the current MRSA population.

Chapter 3: The SCC*mec* typing of MRSA and MR-CoNS isolates according to the method of Kondo et al. 2007

3.1 Introduction

The Milheirico (2007) protocol has been shown to be efficient in screening large numbers of isolates for the most common SCC*mec* types, however many isolates may not fit the patterns produced by this assay and need to be characterized in more detail. This requires each region in the element to be targeted separately, and the method should distinguish differences down to subtype level. One such tool is the SCC*mec* typing assay developed by Kondo et al. (2007). While this protocol is more time-consuming and labour-intensive than other typing methods such as the Milheirico (2007) protocol, it provides much more detailed information about SCC*mec* types and subtypes. The Kondo (2007) protocol includes the use of six multiplex PCRs (mPCRs) which assign *ccr* complex types and *mec* classes, and targets various Open Reading Frames (ORFs) and resistance determinants in the J1, J2 and J3 regions, thereby allowing subtype determination of SCC*mec* types II and IV.

The aim of this study was to characterize the novel and novel variant isolates found in our MRSA and MR-CoNS population in more detail, by optimizing and implementing the Kondo (2007) protocol on a selection of representative isolates.

3.2 Methods

3.2.1 Sample selection

We confirmed the presence of a putative novel and novel variant *SCCmec* type in our MRSA population and were unable to assign *SCCmec* types with the Milheirico (2007) protocol on these isolates. Three representative isolates of both the novel and novel variant types were selected for further investigation using the Kondo (2007) protocol described below. One representative clinical isolate per control was also included, with the exception of *SCCmec* types I and VI, since none were identified in our sample population.

3.2.2 Kondo mPCRs 1 - 5

A collection of multiplex PCRs developed by Kondo et al. (2007) was performed to classify selected isolates into *SCCmec* subtypes. The reference strains used in these PCRs are shown in Table 3.1.

Table 3.1 Reference strains (controls) used in the Kondo protocol

SCCmec type	Reference Strain	mPCR	Origin
I	BAA-38	1 - 3	ATCC
II	BAA-1681	1, 2, 4 - 6	ATCC
IIb	JCSC3063	4	PHRI Tuberculosis Center, Rutgers University [#]
IIA	BK351	4	PHRI Tuberculosis Center, Rutgers University
IIE	RN7170	4	PHRI Tuberculosis Center, Rutgers University
III	BAA-39	1 2, 4 - 6	ATCC
IV	BAA-1680	3	ATCC
IVc	81/108	3	PHRI Tuberculosis Center, Rutgers University
IVd	JCSC4469	3	PHRI Tuberculosis Center, Rutgers University
V	WIS	1 - 2, 4	Medical Microbiology, UCT
VI	BAA-42	1	ATCC

[#] Control strains from PHRO, New Jersey, kindly supplied by Prof Barry Kreiswirth

The reaction mixture of mPCR 1 contained 1 μL of crude DNA template, 0.1 μM of each primer (Integrated DNA Technologies, South Africa) (Table 3.2), 1X KAPA 2G Fast Multiplex Mix and nuclease free water to bring the final reaction volume up to 25 μL . The Veriti thermocycler (Thermo Fisher, USA) was used with an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 2 minutes, annealing at 57°C for 1 minute and elongation at 72°C for 2 minutes. The final elongation step ran at 72°C for 2 minutes. Samples were held at 4°C until amplicons were visualized on 2% SeaKem LE Agarose as described in Chapter 2.

The reaction mixture and cycling parameters of mPCRs 2 to 5 was identical to those used in mPCR 1, with the exception of the annealing step, which occurred at 60°C. PCR products for mPCRs 2 to 5 were visualized on 1.5 % SeaKem LE Agarose gels, as described in Chapter 2, Section 2.2.6.

3.2.3 Kondo mPCR 6

The last mPCR in the Kondo (2007) protocol amplifies the resistance-associated plasmids pT181 and pUB110 which are integrated into the J3 regions found in *SCCmec* types II and III and thus require a long range PCR protocol. We used 1x KAPA Long Range PCR Kit in 25 μL reaction volumes, including 0.3 μM of each primer (Table 3.2) and nuclease free water in a Touch-down PCR run.

Cycling was initiated with a denaturation step at 94°C for 2 minutes, followed by 10 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds and elongation at 68°C for 8 minutes. The next 20 cycles had denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds and elongation at 68°C for 12 minutes. The final elongation step ran at 72°C for 7 minutes. Samples were held at 4°C until agarose gel electrophoresis and amplification

products were visualized on 1.5 % SeaKem LE Agarose gels with the KAPA Universal DNA Ladder (Appendix A), as described in Chapter 2, Section 2.2.6.

Table 3.2 List of primers used for the Kondo (2007) protocol

PCR #	Name	Sequence (5' to 3')	Target	Size (bp)	Concentration
Kondo 1	mA1 (F)	TGCTATCCACCCTCAAACAGG	<i>mecA</i>	286	0.1 µM
	mA2 (R)	AACGTTGTAACCAACCCCAAGA			0.1 µM
	α1 (F)	AACCTATATCATCAATCAGTACGT	<i>ccrA1-ccrB</i>	695	0.1 µM
	α2 (F)	TAAAGGCATCAATGCACAAACACT	<i>ccrA2-ccrB</i>	937	0.1 µM
	α3 (F)	AGCTCAAAAGCAAGCAATAGAAT	<i>ccrA3-ccrB</i>	1791	0.1 µM
	βc (R)	ATTGCCTTGATAATAGCCITCT	See above		0.1 µM
	α 4.2 (F)	GTATCAATGCACCAGAACTT	<i>ccrA4-ccrB4</i>	1287	0.1 µM
	β 4.2 (R)	TTGCGACTCTCTTGCGGTTT			0.1 µM
Kondo 2	Υ F	CCTTTATAGACTGGATTATTCAAAATAT	<i>ccrC</i>	518	0.1 µM
	Υ R	CGTCTATTACAAGATGTAAAGGATAAT			0.1 µM
	ml6 (R)	CATAACTTCCCATTCTGCAGATG	<i>mecA-mecI</i>	1963	0.1 µM
	IS7 (R)	ATGCTTAATGATAGCATCCGAATG	<i>mecA-IS1272</i>	2827	0.1 µM
	IS2(iS-2) (R)	TGAGGTTATTCAGATATTTTCGATGT	<i>mecA-IS431</i>	804	0.1 µM
	mA7 (F)	ATATACCAAACCCGACAACACTACA	See above		0.1 µM
	1a3 (F)	TTTAGGAGGTAATCTCCTTGATG	E007	154	0.1 µM
	1a4 (R)	TTTTGCGTTTGCATCTCTACC			0.1 µM
Kondo 3	4a1 (F)	TTTGAATGCCCTCCATGAATAAAAT	CQ02	458	0.1 µM
	4a3 (R)	AGAAAAGATAGAAGTTCGAAAGA			0.1 µM
	4b3 (F)	AACCAACAGTGTTACAGCTT	M001	726	0.1 µM
	4b4 (R)	CGGATTTTAGACTCATCACCAT			0.1 µM
	4c4 (F)	AGGAAATCGATGTCATTATAA	CR008	259	0.1 µM
	4c5 (R)	ATCCATTTCTCAGGAGTTAG			0.1 µM
	4d3 (F)	AATTCACCCGTACCTGAGAA	CD002	1242	0.1 µM
	4d4 (R)	AGAATGTGGTTATAAGATAGCTA			0.1 µM

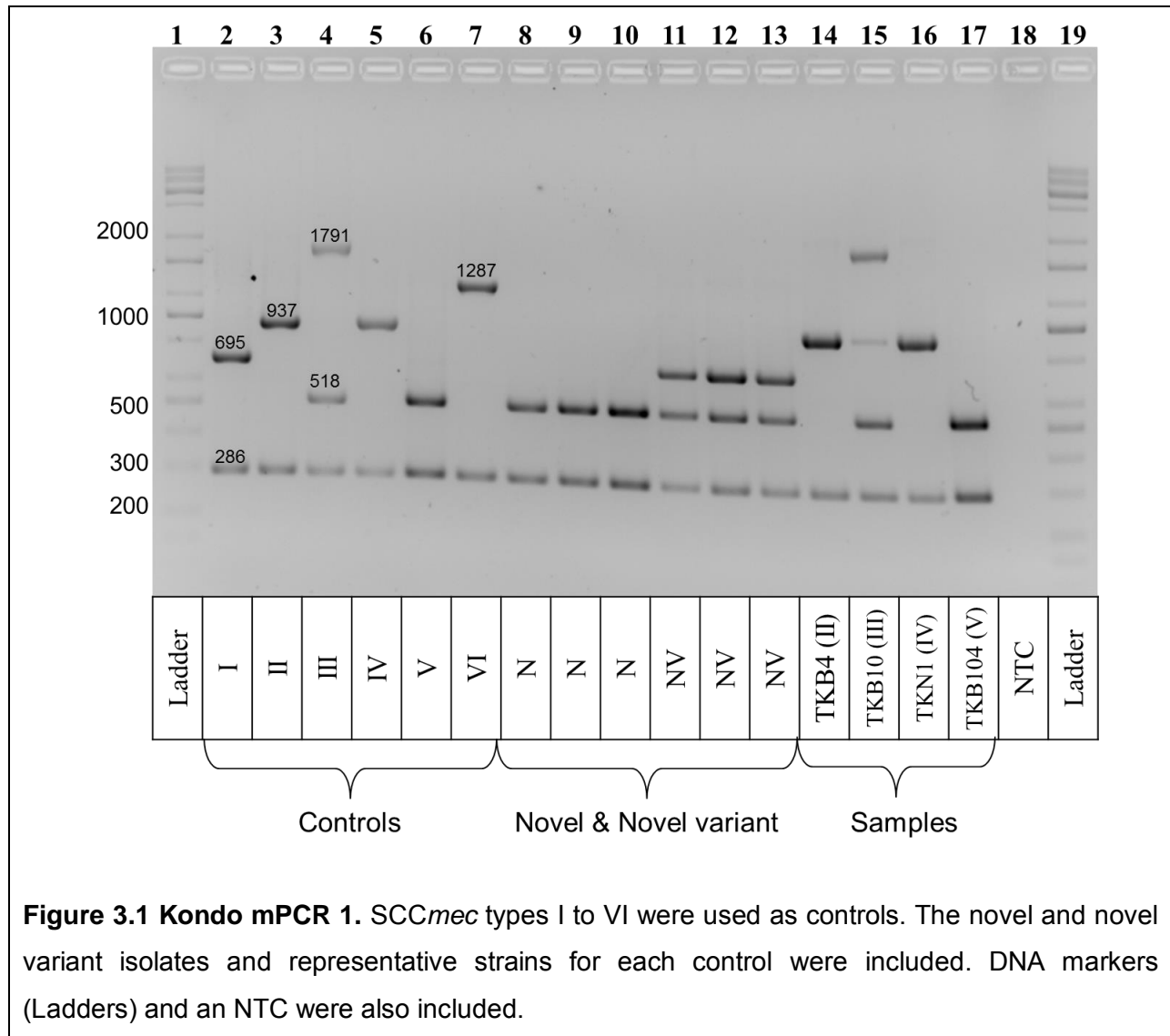
Table 3.3 List of primers used for the Kondo (2007) protocol (continued)

Kondo 4	kdpB1 (F)	GATTACTTCAGAACCAGGTCAT	<i>kdpB</i>	287	0.1 μ M
	kdpB2 (R)	TAAACTGTGTCACACGATCCAT			0.1 μ M
	2b3 (F)	GCTCTAAAAGTTGGATATGCG	SA01	1518	0.1 μ M
	2b4 (R)	TGGATTGAATCGACTAGAATCG			0.1 μ M
	4b3 (F)	AACCAACAGTGGTTACAGCTT	IIE03 or	726	0.1 μ M
	4b4 (R)	CGGATTTTAGACTCATCACCAT	M001		0.1 μ M
	II4-3 (F)	GTACCGCTGAATATTGATAGTGAT	RN06	2003	0.1 μ M
	II4-1 (R)	ACTCTAATCCTAATCACCGAAC			0.1 μ M
	3a1 (F)	ATGGCTTCAGCATCAATGAG	Z004	503	0.1 μ M
	3a2 (R)	ATATCCTTCAAGCGCGTTTC			0.1 μ M
Kondo 5	5a1 (F)	ACCTACAGCCATTGCATTATG	V024	1159	0.1 μ M
	5a2 (R)	TGTATACATTTGCCACTAGCT			0.1 μ M
	ermA1 (F)	TGAAACAATTTGTAACATTGA	<i>ermA</i> - CN030 or CZ021	2756	0.1 μ M
Kondo 5	cad4 (F)	ATTGCGATTCTTTCCGATATGG	<i>cadB</i> - CN030 or CZ021	1540	0.1 μ M
	mN5 (R)	TTGCTTCGGGACTTACCTCTAGT	See above		0.1 μ M
Kondo 6	ant1 (R)	CAGACCAATCAACATGGCACC	<i>mecA</i> - <i>ant</i> (4')	4952	0.3 μ M
	pT181-2 (R)	AGGTTTATTGTCACTACAATTGA	<i>mecA-tetK</i>	7406	0.3 μ M
	mA1 (F)	TGCTATCCACCCTCAAACAGG	See above		0.3 μ M

3.3 Results

3.3.1 Kondo mPCR 1

Kondo mPCR 1 typed the various *ccr* class combinations found in the *SCCmec* element (Figure 3.1).



The novel type (Lanes 8 – 10) was shown to carry *ccrC*, which is characteristic of types V and VII. The novel variant (Lanes 11 – 13) had a combination of bands representing *ccrC* and

ccrA1B1. The isolates we included from our sample population (Lanes 14 – 17) all corresponded to their respective controls, with the exception of the type III isolate (Lane 15), which had a faint band matching *ccrA2B2*. The PCR was repeated and the same result was found, ruling out sample carry-over between wells during the loading of the gel, and possibly indicating that the isolate in question is a variant.

3.3.2 Kondo mPCR 2

Figure 3.2 shows the results of the *mec* class assignment that was done using Kondo mPCR 2.

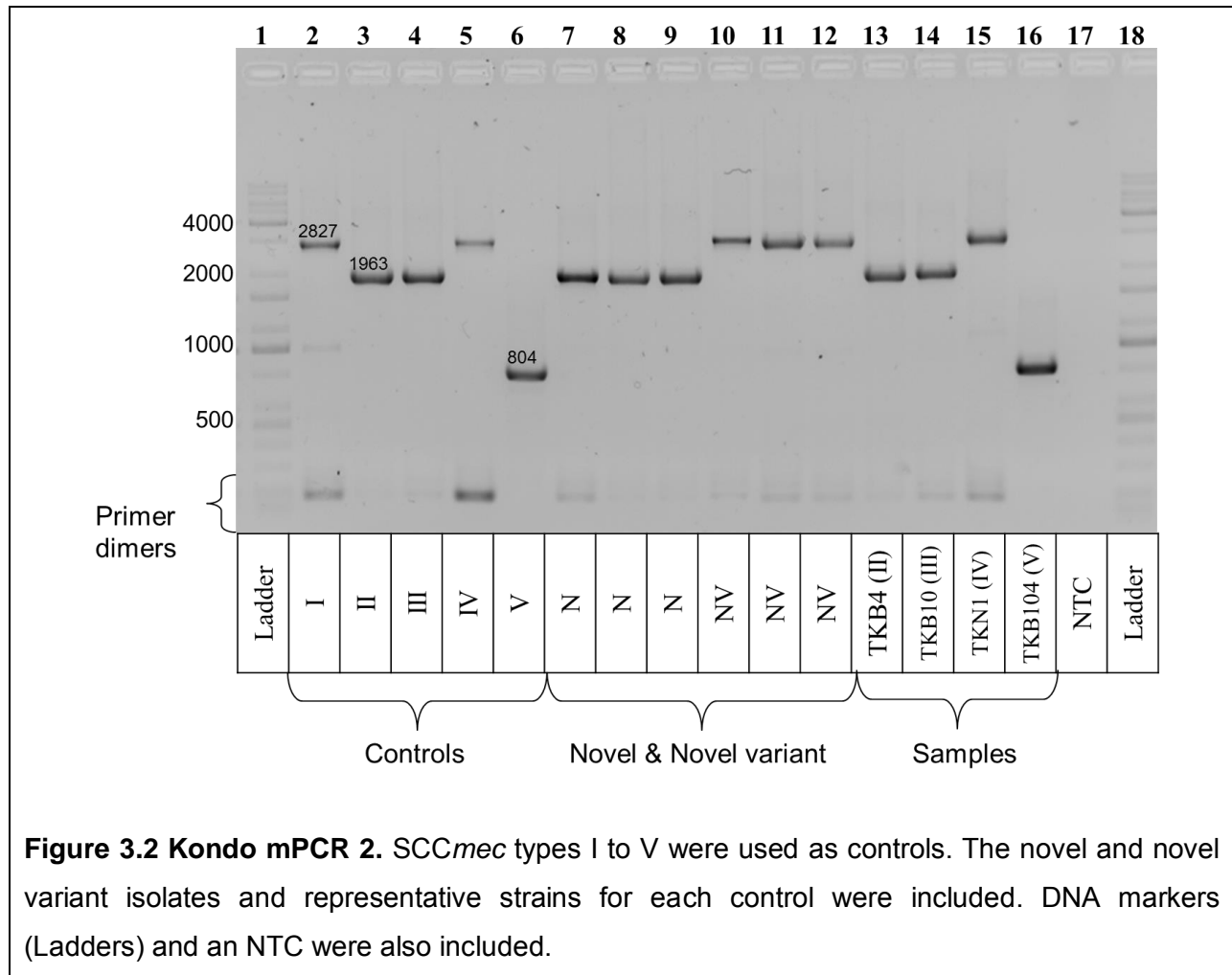


Figure 3.2 Kondo mPCR 2. SCC*mec* types I to V were used as controls. The novel and novel variant isolates and representative strains for each control were included. DNA markers (Ladders) and an NTC were also included.

Faint primer dimers can be seen at the bottom of the gel, however these do not impact on the results as they are very small and easily distinguishable from the rest of the products. The novel type isolates (Lanes 7 – 9) had class A *mec* genes and the novel variant type (Lanes 10 – 12) had class B *mec* genes. The clinical isolates (Lanes 13 – 16) correspond to their respective controls. The combined results of mPCRs 1 and 2 confirm the results of the Milheirico (2007) protocol: the novel variant resembles SCC*mec* type I, with the addition of *ccrC* (*ccrA1B1C* /class B *mec*) and the novel type has a previously unseen combination (*ccrC* /class A *mec*).

3.3.3 Kondo mPCR 3

This PCR inspected ORFs present in the J1 regions of SCC*mec* types I, IV, IVb, IVc and IVd (Figure 3.3).

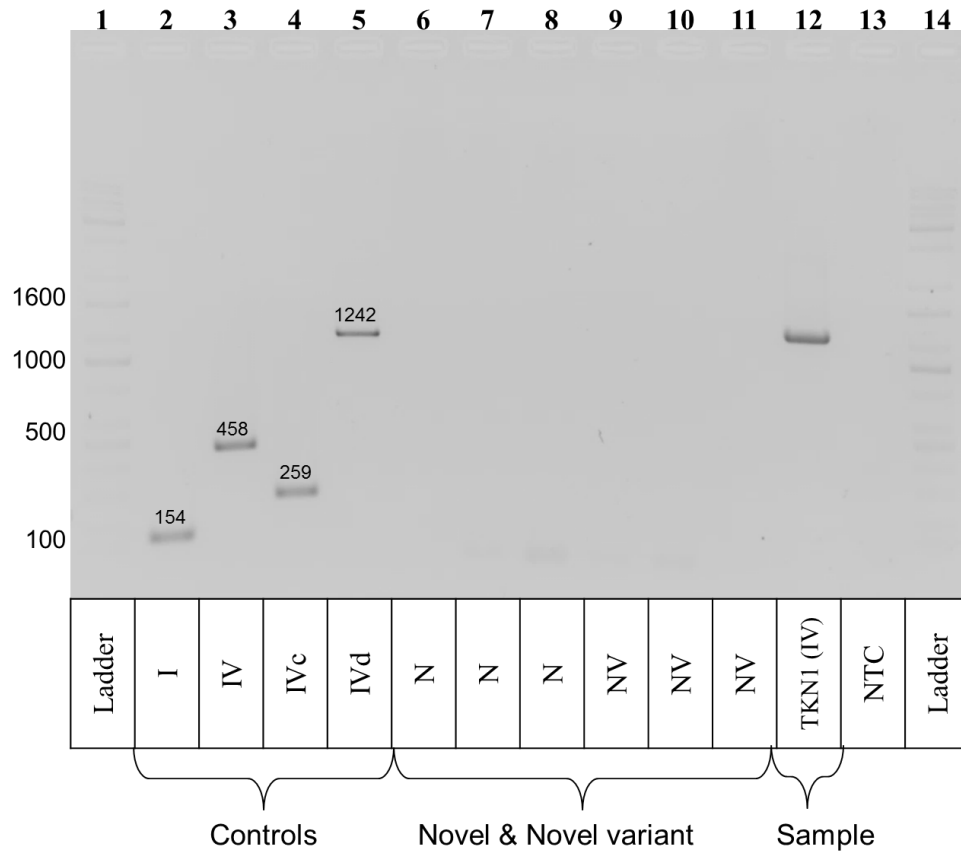
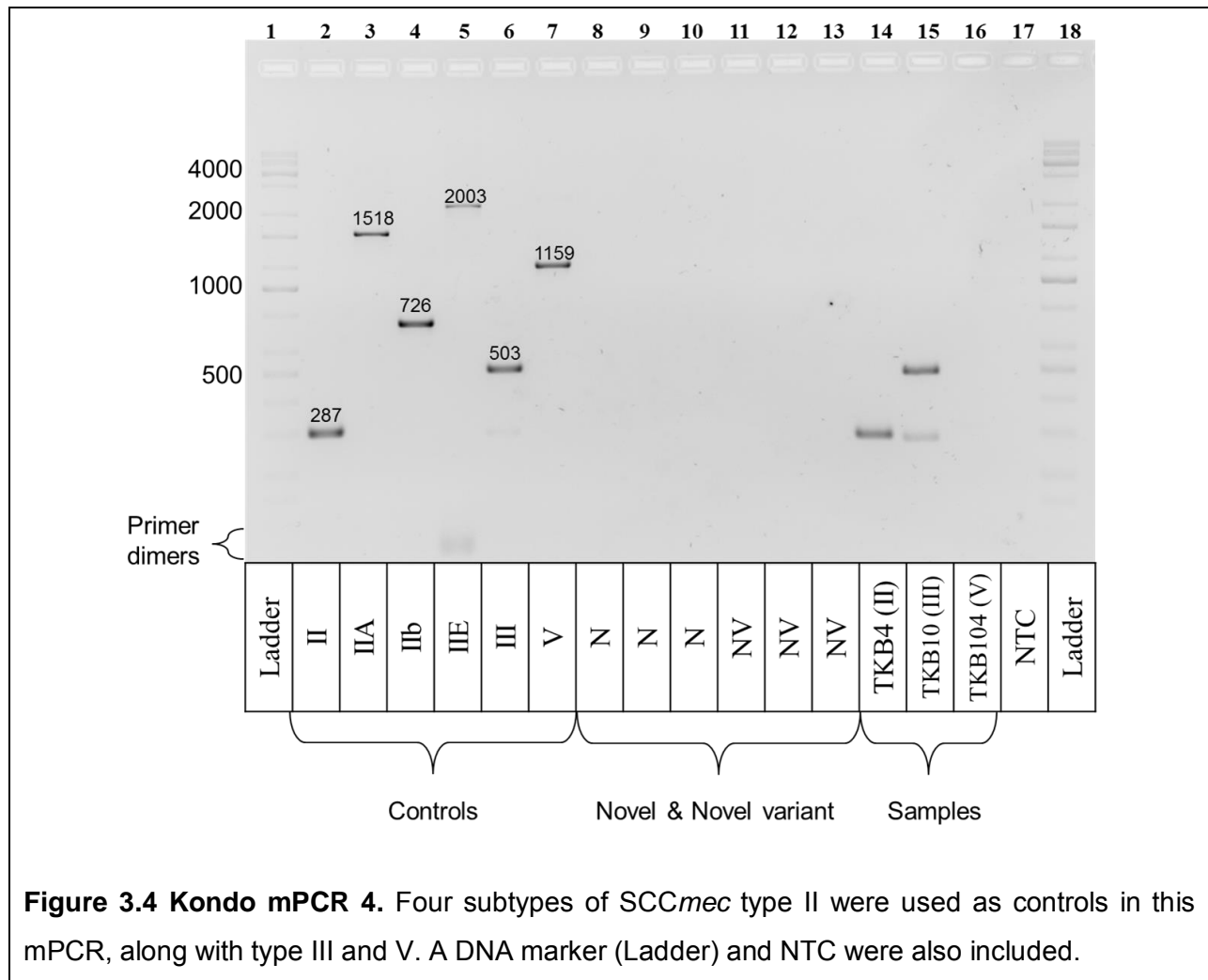


Figure 3.3 Kondo mPCR 3. SCCmec types I and three subtypes of IV were the control strains in this mPCR. The novel and novel variant isolates and representative strain for control IV were included. DNA markers (Ladders) and an NTC were also included.

The control for type IVb could not be obtained for this study, however this is unlikely to impact on the results since the expected target (726 bp) was not observed in any of the isolates tested. Neither the novel nor the novel variant types (Lanes 6 – 11) had any amplification products, indicating that they do not share J1 region ORFs with types I or IV. The representative type IV isolate (Lane 12) from our population corresponds to subtype IVd.

3.3.4 Kondo mPCR 4

Kondo mPCR4 targeted ORFs present in the J1 regions of SCC*mec* types II, IIb, IIA, IIE, III and V (Figure 3.4).



Just as seen in mPCR3, the novel and novel variant types (Lanes 8 – 13) do not share any of the targeted J1 region ORFs with the SCC*mec* reference strains used. This correlates with results from the Milheirico (2007) protocol that show the novel type does not amplify the J1 region target (Figure 2.5). The type III isolate from our population (TKB10, Lane 15) amplified the expected target (503 bp) as well as the type II target (287 bp). The PCR was repeated and

gel re-run to rule out carry-over between samples, with the same result. The representative *SCCmec* type V isolate from our population (Lane 16) and the control strain BAA-1688 (not shown) also had no amplification of the 1159 bp product associated with the type V J1 region. This supports the results from the Milheirico (2007) protocol that suggest it is a type V variant.

3.3.5 Kondo mPCR 5

Two transposons commonly associated with *SCCmec* types II and III, Tn554 and Ψ Tn554, were targeted in this mPCR (Figure 3.5).

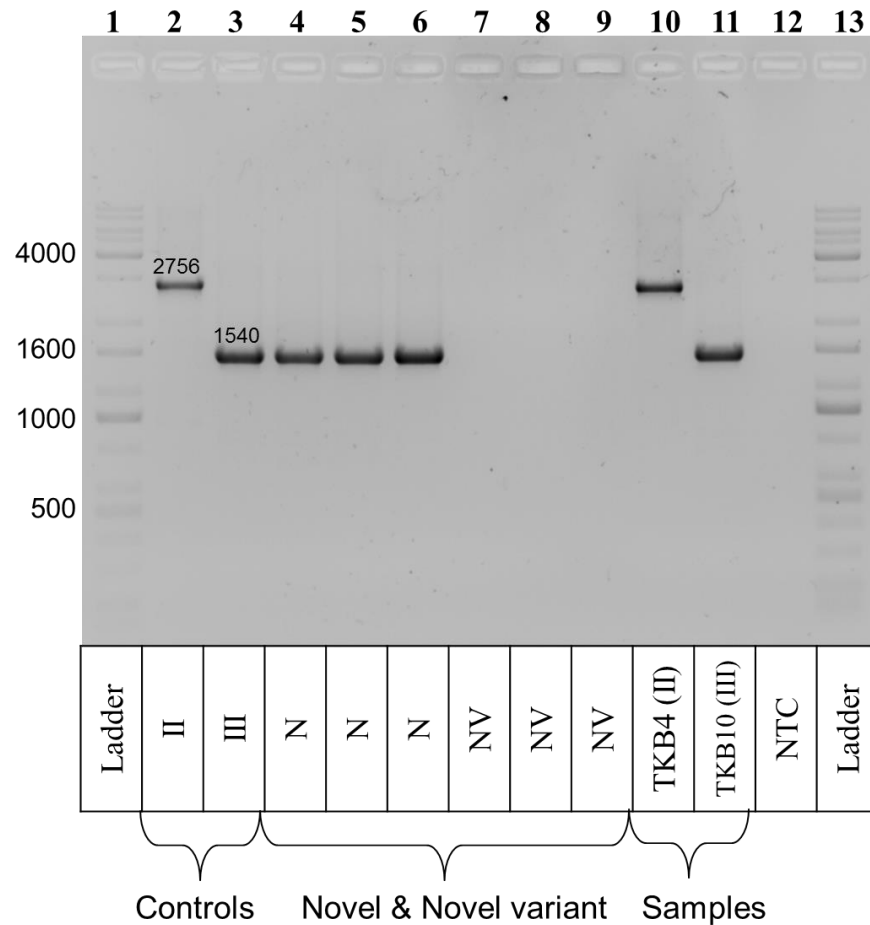
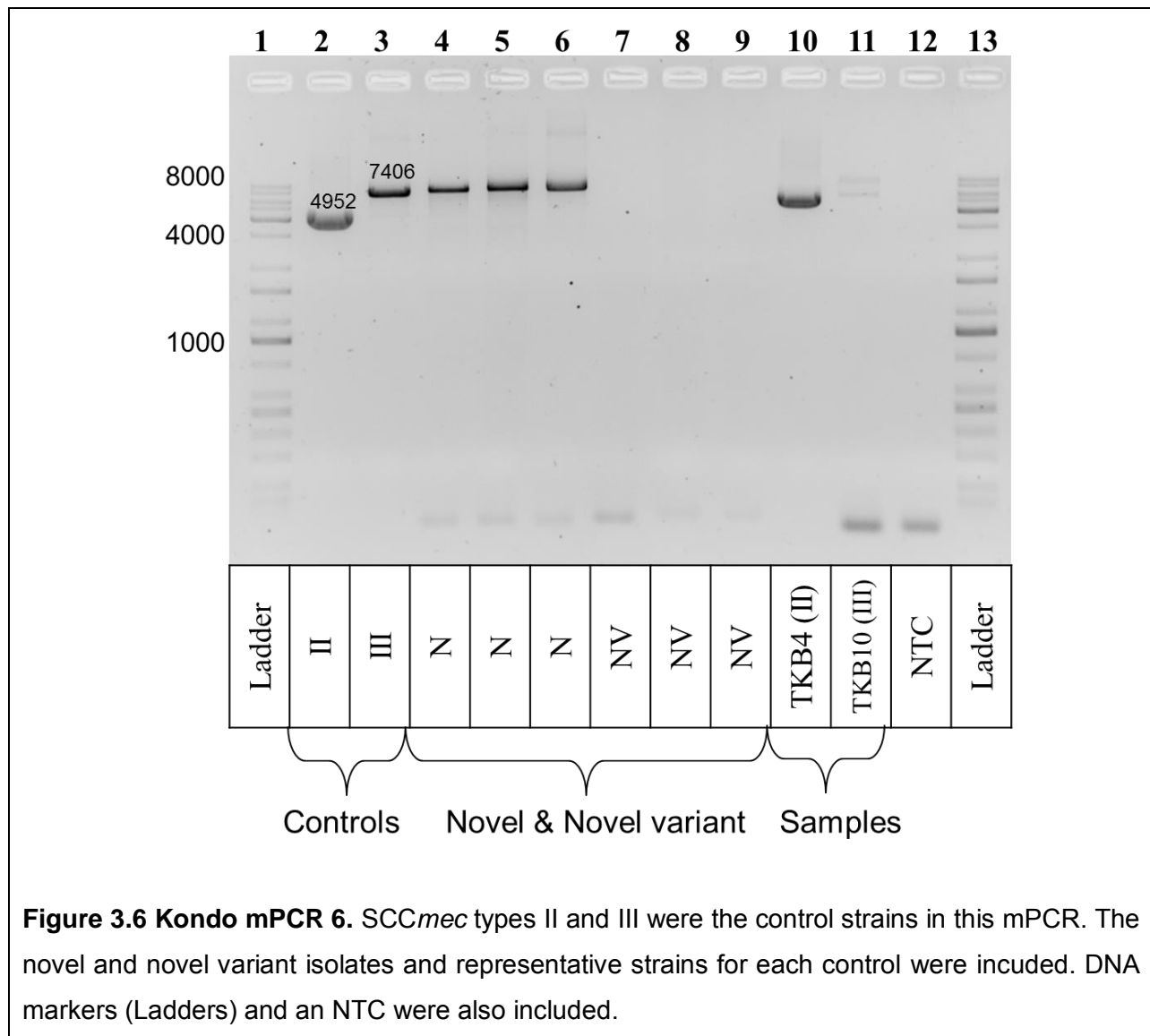


Figure 3.5 Kondo mPCR 5. *SCCmec* types II and III were the control strains in this mPCR. The novel and novel variant isolates and representative strains for each control were included. DNA markers (Ladders) and an NTC were also included.

The novel variant SCC*mec* type (Lanes 7 – 9) did not have any amplification, but the novel type (Lanes 4 – 6) was found to contain the 1540 bp product targeting Ψ Tn554, which is associated with cadmium resistance. The clinical isolates we included (Lanes 10 – 11) harbour the expected resistance-associated transposons related to their SCC*mec* types.

3.3.6 Kondo mPCR 6

Figure 3.6 shows the results of a long-range PCR which targeted resistance-associated plasmids found in the J3 regions of some SCC*mec* elements.



pUB110 (4952 bp), which confers resistance to bleomycin and tobramycin, was not present in the novel or novel variant types (Lanes 4 – 9). pT181 (associated with tetracycline resistance, 7406 bp) was found in the novel type (Lanes 4 -6). The clinical isolates representing type II (Lane 10) amplified a target that appears slightly larger than the control, however since the products are so large, it is likely that this is due to uneven DNA separation across the gel. Amplification for pT181 in the type III isolate (Lane 11) was extremely faint, possibly indicating either a very low copy level of the plasmid or the absence of the plasmid. A second band could also be observed for this isolate, but it is unclear whether this is true amplification or non-specific bands. The absence of this plasmid may indicate that this isolate is a type IIIA variant.

3.4 Discussion

3.4.1 Characterization of the novel SCC*mec* type

The combination of the Milheirico (2007) and Kondo (2007) protocol results show that the putative novel SCC*mec* type found in our MRSA isolates contains an unpublished *ccr* complex-*mec* complex combination. The *ccr* class found in this type is characteristic of types V, VII and the SCC_{Hg} element (Ito et al. 2004; Chongtrakool et al. 2006; Berglund et al. 2008), and the *mec* class of types II, III and VIII (Ito et al. 2001; Zhang et al. 2009). It is improbable that the novel element was formed via any recombination event including types V, VII and VIII, since only one type V isolate was identified in our setting, and type VII and VIII were not present. It is therefore expected that the *ccrC* class seen in the novel type originated from a SCC_{Hg} element. The SCC*mec* III isolates in our population also amplified *ccrC*, indicating that they possibly carried the SCC_{Hg} element. The combination of type III and SCC_{Hg} is frequently reported (Chongtrakool et al. 2006). However, the type III isolate from our population did not amplify the target for Tn554 which is present in SCC_{Hg} but rather showed faint amplification of regions corresponding to type II. To our knowledge, the combination of *ccr* types 2 and 3 (*ccrA2ccrB2*, *ccrA3ccrB3*) has been reported in MR-CoNS specimens, but not in MRSA (Sani et al. 2014). Further genomic analysis is required to determine the true structure of these SCC elements.

Furthermore, our results show that the novel type contained a resistance-associated transposon and a resistance-associated plasmid, both associated with SCC*mec* type III. Interestingly, many of the novel type isolates were grouped in the same PFGE pulsotypes as the type III isolates, indicating further genotypic similarity between the two types (Karayem 2014). There was no amplification in any of the J1 region targets in the novel type, indicating a possible deletion or sequence change in the J1 region.

3.4.2 Characterization of the novel variant SCC*mec* type

The novel variant SCC*mec* type found in our MRSA population had a similar banding pattern to SCC*mec* type I using the Milheirico (2007) protocol and this was confirmed using the first two multiplex PCRs of the Kondo (2007) protocol. This variant carried a combination of a class B *mec* and *ccrA1B1* as well as the same *ccrC* class found in the novel type. No other amplification products were found in any of the joining regions; therefore it is unclear if the novel variant shares similarity with other SCC*mec* types in our setting. The addition of *ccrC* to this element may be an indication that it has also acquired SCC_{Hg}, just as seen with the novel SCC element; but typing showed that a characteristic transposon carried by SCC_{Hg}, Tn554, was not present. This suggests that the SCC_{Hg} element may be truncated, or that *ccrC* was acquired by recombination with the other SCC*mec* elements in our setting that carry it. The fact that the novel, novel variant and SCC*mec* III elements in our population contain *ccrC* may indicate that it, or the element it is likely carried on, is easily transferred between SCC*mec* types or that it conveys a fitness advantage to MRSA isolates in a clinical setting.

3.4.3 Conclusion

The Kondo (2007) protocol confirmed the results of SCC*mec* typing with the Milheirico (2007) protocol and showed that our MRSA population contained isolates with unique SCC*mec* elements. Novel combinations of the main gene complexes, *ccr* and *mec* were identified in our novel and novel variant SCC*mec* types. Furthermore, the Kondo (2007) protocol allowed us to detect the presence or absence of other important resistance determining elements in these novel types. The J1 regions in both the novel and novel variants need to be inspected further to determine whether there has been a deletion or if it has been changed in such a manner that the PCR primers cannot detect it. The Kondo (2007) protocol provides the best discriminatory power of the SCC*mec* typing assays and is the typing method recommended by the IWG-SCC (<http://www.staphylococcus.net>). However, while it provides a great deal of insight into the

composition of novel SCC elements, sequencing the entire length of these elements is essential in order to elucidate and confirm their structure (Ito et al. 2009).

Chapter 4: Whole Genome Sequencing

4.1. Introduction

The rapidly growing diversity of SCC*mec* types, especially those present in MR-CoNS isolates, presents significant challenges for conventional SCC*mec* typing assays such as mPCR. The reason is that changes in genetic structure, such as insertions and deletions (indels) and single nucleotide polymorphisms (SNPs) may influence the ability of these mPCRs to effectively target and amplify important regions. While most SCC*mec* typing assays can assist in identifying possible novel types, sequencing of the elements is necessary to fully characterize their structures.

Recent advances in next-generation sequencing (NGS) and increased availability of NGS technology have made it an ideal tool for genotypic analysis. NGS generates large amounts of data that can be assembled *de novo* to detect novel regions and aligned against closely related reference sequences to detect SNPs and indels.

We submitted a selection of isolates for whole genome sequencing using NGS Illumina technology, to characterize the structures of the putative novel and novel variant SCC elements identified in our setting.

4.2. Methods

4.2.1. Sample selection

We selected 11 MRSA isolates and one MR-CoNS (*S. epidermidis*) isolate for whole genome sequencing, based on their *SCCmec* typing results with the Milheirico (2007) and Kondo (2007) protocols. We also considered results from other epidemiological tests such as PFGE, *spa* typing and MLST (as determined previously by Karayem et al., 2014). It was noted that all of the novel *SCCmec* type isolates clustered in pulsotypes that also contained *SCCmec* type III isolates and that these isolates (with both the novel element and *SCCmec* III) shared the same *spa* type, t037 and sequence type (ST), ST239. Therefore five *SCCmec* type III isolates were selected for WGS analysis, to compare the structure of the *SCCmec* type III elements to those found in the novel type (Table 4.1).

Table 4.1 Representative samples chosen for WGS

Sample Name	Sequencing Name	<i>SCCmec</i> type
TKB-8	M8	Novel variant
TKB-16	M16	Novel variant
TKB-10	M10	Novel
TKB-11	M11	III
TKB-14	M14	Novel
TKB-108	M108	III
TKB-105	M105	Novel
TKB-106	M106	III
TKB-77	M77	Novel
TKB-156A	M156A	III
TKB-156B	M156B	III
Co8 (CoNS)	MCo8	Novel

4.2.2. DNA extraction

Commercial DNA extraction kits failed to produce sufficient quantities of quality DNA, therefore an in-house phenol-chloroform DNA extraction optimized for *Mycobacterium tuberculosis* (Warren 2016) was adapted and used to extract DNA from the staphylococcal isolates. All media and buffer compositions are listed in Addendum A. Briefly; blood agar plates (NHLS Media Laboratory) were inoculated with a single microbank bead and incubated at 37°C for 18 – 24 hours. A loopful of colonies was suspended in sterile saline solution and used to culture a full lawn of growth on two tryptic soy broth (TSB) agar plates (Addendum A) per isolate at 37°C for 24 hours.

50 ml polypropylene tubes (Falcon tubes) were prepared with 12mL of DNA extraction buffer (pH 7.4) (Addendum A) and approximately 20x 5mm glass balls. Sterile loops were used to scrape off and transfer the culture to the Falcon tubes in a class II laminar flow cabinet. The Falcon tubes were then vortexed until the mixtures were homogenized and no clumps were observed. 60 µL of a cell lysis agent, lysostaphin (10 mg/mL) (Sigma-Aldrich, South Africa), was added to each tube and then mixed by gentle inversion and incubated at 37°C for 30 minutes. 600 µL of 10X proteinase K buffer (pH 7.8) (Addendum A) and 300 µL of proteinase K (10 mg/ml) (Qiagen, Germany) were added to each tube, gently mixed by inversion and incubated overnight at 42°C. Proteinase K inactivates any nucleases released during cell lysis that may degrade the DNA in the buffer.

10 mL of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the Falcon tube in a fume hood, and gently mixed by inversion every 30 minutes for 2 hours. This was done to separate the samples into phases, based on their solubility in water. The tubes were then centrifuged at 3000 x *g* for 20 minutes after which the top aqueous phase containing the DNA was carefully removed and placed in a sterile Falcon tube. The phenol/chloroform/isoamyl alcohol step and centrifugation was repeated, without the 2 hour incubation, to ensure clean separation of the

DNA from other viscous cell materials. The top aqueous phase was aspirated into a tube containing 10 mL chloroform/isoamyl alcohol (24:1), mixed gently and centrifuged at 3000 x *g* for 20 minutes. This step removes any traces of phenol and minimizes the negative effects it might have on downstream WGS analysis. The top phase was again carefully aspirated into sterile Falcon tubes containing one tenth volume 3M sodium acetate (pH 5.2) (Sigma-Aldrich, South Africa) to change the charge and pH of the DNA, thereby making it more hydrophobic and easier to precipitate.

One volume of ice cold 100% isopropanol was added slowly with minimal movement to avoid premature precipitation which leads to the presence of unwanted compounds. The Falcon tubes were inverted slowly three to four times to precipitate the DNA, which was immediately fished out of the solution using sterile glass rod hooks. These rods with the DNA were placed in microcentrifuge tubes containing 1 mL 70% ethanol and incubated at room temperature (25°C) for 10 minutes to remove excess salts. The rods were then transferred to sterile 1.5 mL microcentrifuge tubes and incubated at room temperature until all the ethanol had evaporated.

Once dry, the DNA was rehydrated in TE (Tris-EDTA) buffer (pH 8.0) (Addendum A) and released from the rods by gentle mixing and placed in an incubator at 65°C for one hour. The EDTA in this buffer chelates metal cations in the sample and effectively inactivates any DNA-degrading nucleases still present. The amount of TE buffer added to each sample varied from 300 to 600 µL, depending on the amount of DNA extracted and the viscosity of the sample.

The DNA concentration was measured spectrophotometrically with a BioDrop µLite (Whitehead Scientific, Cape Town) to assess A280/260 and A260/230 ratios. Additional TE buffer was added to particularly viscous samples that were difficult to pipette. The A280/260 ratio for pure DNA is expected to fall between 1.8 and 2.0 and values lower than this may be an indication of contaminants that absorb at 260 nm, such as phenol. The A260/230 values are expected to range between 2.0 to 2.2 and lower values may indicate the presence of contaminants that

interfere with sequencing. However, values as low as 1.5 have been found to be adequate for sequencing.

4.2.3. Quality Control of extracted DNA

To ensure sufficient quantities and quality of starting material, various quality control tests were conducted by the Centre for Proteomic and Genomic Research (CPGR) prior to sequence library preparation. The DNA concentration of each sample was spectrophotometrically measured for a second time with the Nanodrop ND 1000 (Thermo Scientific, USA).

To determine the absolute dsDNA concentration, fluorometric measurement was done with the Qubit dsDNA BR assay on a Qubit 2.0 fluorometer. The integrity of the genomic DNA was evaluated by 2% gel electrophoresis to look for high molecular weight DNA (larger than 10 kb).

4.2.4. DNA library preparation and cluster amplification

The Nextera® XT DNA Sample Preparation Kit (Illumina, South Africa) was used to create libraries for sequencing, following the manufacturer's protocol (Addendum B). Briefly, input DNA was simultaneously fragmented and tagged (tagmented) with end-adapter sequences by the Nextera XT transposome. Tagmented DNA was then amplified by a multiplex PCR which added unique adapters and other sequences required for cluster formation. Following amplification, the samples were barcoded with unique combinations of indices to allow sample pooling during sequencing (Table 4.2).

Table 4.2 Barcodes (indices) applied during WGS

Indices	N701 TAAGGCGA	N702 CGTACTAG	N703 AGGCAGAA	N704 TCCTGAGC
S507 AAGGAGTA	M11	M10	M77	M156A
S508 CTAAGCCT	M156B	M14	M108	M16
S517 GCGTAAGA	M8	M105	M106	MCo8

Finally, the library DNA was purified with AMPure XP beads (Beckmann Coulter), to remove very short library fragments, thereby improving the quality of subsequent sequencing reactions. Equal volumes of normalized library were combined and diluted in hybridization buffer, and heat denatured. The libraries were then loaded onto the MiSeq Reagent Kit v3 (Illumina) cartridges following the manufacturer's instructions.

4.2.5. MiSeq Illumina sequencing

The clonal clusters used for sequencing were generated via bridge amplification (Figure 4.1).

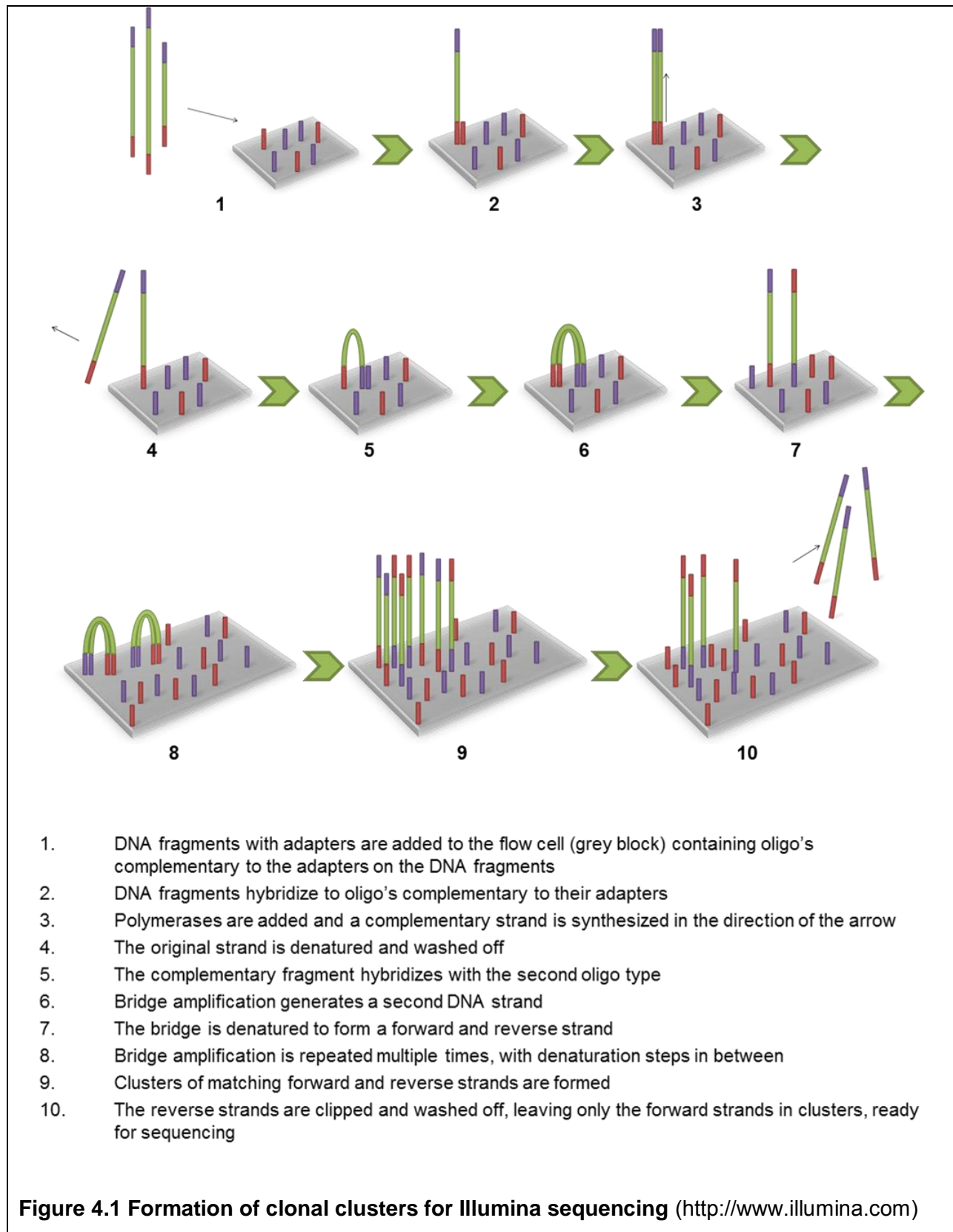
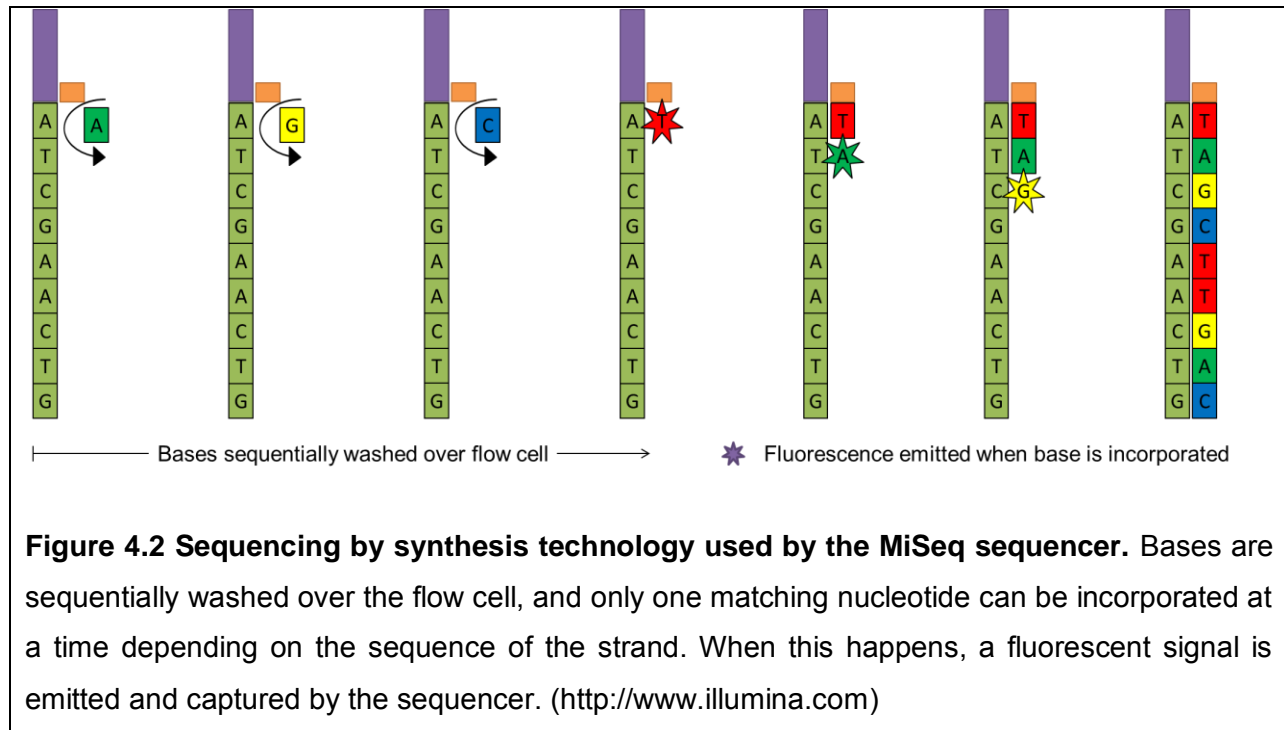


Figure 4.1 Formation of clonal clusters for Illumina sequencing (<http://www.illumina.com>)

The MiSeq sequencer used Illumina's sequencing by synthesis (SBS) technology to sequence the clusters (Figure 4.2).



The fluorescence generated by fluorophore labeled reversible terminators was measured. These terminating dNTPs block polymerization, causing only a single base to be incorporated at a time and therefore allowing accurate base-by-base sequencing results. Every cluster is imaged simultaneously, thereby generating massively parallel sequencing data. After imaging, the terminators were cleaved to allow incorporation of the following base.

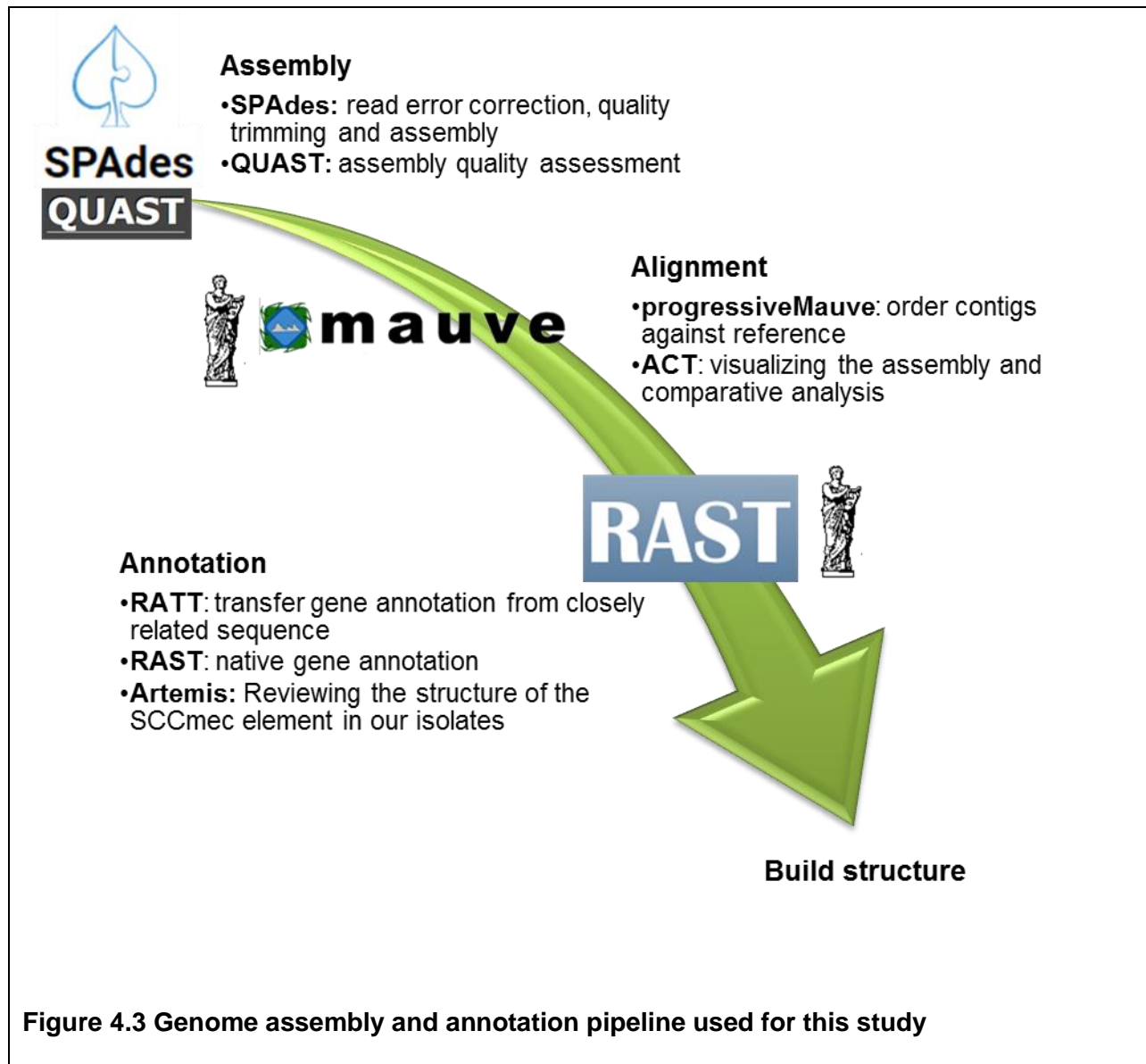
After the forward strands were sequenced, the reverse strands were reformed by a single bridge amplification step, followed by clipping of the forward strands. The sequencing process was then repeated for the reverse strand. Since each sample was barcoded with unique indices, the resulting forward and reverse reads of each sample can be identified and paired together for downstream analysis.

4.2.6. Sequence quality analysis

For the 600 cycle MiSeq reagent kit v3, sequencing data output is expected to be as high as 15 Gb with 70% or more of the data passing a quality (Phred) score of 30 (Q30). Q scores are logarithmically related to base call error probability, for example Q30 is equivalent to 1 incorrect base call per 1000 bases (99.9% accuracy) and Q20 is equivalent to 1 error per 100 bases (99% accuracy rate). Low Q scores will result in false-positive variant calls in downstream analysis, which makes data untrustworthy. The gold standard for read quality in next-generation sequencing analysis is Q30, and reads below this value should preferably be filtered out during analysis. In addition to the quality score summary provided with the data, each sequence file was also subjected to an individual and comprehensive quality control (QC) check using FastQC version 0.10.1 (Babraham Bioinformatics, UK). FastQC provided information about the sequence quality, sequence content, GC content, sequence length distribution and sequence duplication levels for each individual sequence file.

4.2.7. Genome assembly and annotation

Figure 4.3 summarizes the genome assembly and annotation pipeline that was used.



De novo genome assembly was performed using the St. Petersburg genome assembler (SPAdes v3.6.0, Algorithmic Biology Lab) (Bankevich et al. 2012) through the online service at BaseSpace (Illumina, USA). Samples that contained more than 1.00 gigabases of data (SPAdes online application limit) were run with SPAdes on a command line basis. Parameter specifications for the command line tools used in this pipeline can be found in Addendum B: Supplementary Methods.

The SPAdes assembler produced contigs (contiguous DNA sequences of overlapping reads) in *fasta* format, ready for downstream analysis. The quality of the assembly was assessed using QUAST (Gurevich et al. 2013) to produce statistics including GC content (%), the total length of the assembly, the number of contigs and the N50 value, which are important markers for assembly quality. There are no quality cut-offs for these values, however it is generally accepted that fewer contigs (<200) and high N50 values (>10000) are indications of good assemblies. The N50 value indicates that 50% of the contigs in the assembly are the size (in bp) of the N50 value or larger, therefore a high N50 value indicates that most of the contigs are large and that assembly was efficient. The QUAST quality report was automatically generated using the online SPAdes application and manually generated through a web-based utility (<http://quast.bioinf.spbau.ru/>) for the command line SPAdes assemblies.

The contigs generated by SPAdes were ordered against closely related reference sequences from the NCBI online repository. JKD6008 (accession NC_017341) contained a *SCCmec* type III region and was therefore used as reference for the novel and type III isolates. COL (accession NC_002951) contained a *SCCmec* type I region and was used as reference for the novel variant isolates. A methicillin resistant *S. epidermidis* (MRSE) genome, ATCC 12228 (accession NC_004461) was chosen as reference for the *S. epidermidis* isolate, since it has been used as an MRSE reference genome in a South African setting before (Biswas et al. 2015). The expected genome sizes for the MRSA isolates was between 2.8 and 2.9 Gb and the MRSE isolate was expected to have a genome of 2.4 to 2.5 Gb. The alignment was done using the “Move Contigs” function of the progressive Mauve multiple genome alignment tool (Darling lab, Sydney) (Darling et al. 2010). A comparison between the reference sequence and the newly ordered contigs was generated using command line tools. The alignment was then visualized with the Artemis Comparison Tool (ACT, Wellcome Trust Sanger Institute, UK) (Carver et al. 2005) to inspect synteny and homology between the genomes.

We transferred the annotation from our reference sequences to our ordered contigs using the Rapid Annotation Transfer Tool (RATT) (Otto et al. 2011). Areas without synteny, i.e. those unique to the ordered contigs, were annotated manually with BLAST searches through the Artemis DNA viewer and annotation tool (Rutherford et al. 2000). We also annotated a subset of isolates automatically with Rapid Annotation using Subsystem Technology (RAST) v2.0 (Aziz et al. 2008) at <http://rast.nmpdr.org/rast.cgi> to confirm the annotation done via RATT and manually.

4.3. Results

4.3.1. DNA extraction

The extracted DNA was noted to be very viscous in all twelve samples, due to the high concentration of DNA present. Samples that were difficult to work with were diluted with additional TE buffer before spectrophotometric analysis. Spectrophotometers (including the BioDrop μ Lite) are known to overestimate the amount of DNA; therefore, our samples were diluted to 100 ng/ μ L in 100 μ L of TE buffer to ensure that sufficient DNA concentrations (30 ng/ μ L) were provided for whole genome sequencing.

4.3.2. Quality Control of extracted DNA

All samples passed QC and showed no significant deviations. The A280/260 ratios of all samples ranged between 1.86 and 2.05, indicating high purity DNA and the A260/230 values were between 1.73 and 3.01. Qubit analysis provided accurate DNA concentration measurements, ranging between 30.9 and 59.9 ng/ μ L, with the exception of a single sample that had an unusually high concentration (620 ng/ μ L). This sample was noted to be extremely viscous, and it is likely that the viscosity affected the accuracy of the initial BioDrop reading. All twelve samples had intact genomic DNA (gDNA) and only contained minor single stranded DNA (ssDNA) and RNA contaminants which do not interfere with the library preparation assay (Addendum A).

4.3.3. Sequence quality analysis

The total sequencing data output was 11.41 Gb with 68.3% of all sequences at or exceeding Q30 scores (Figure 4.4).

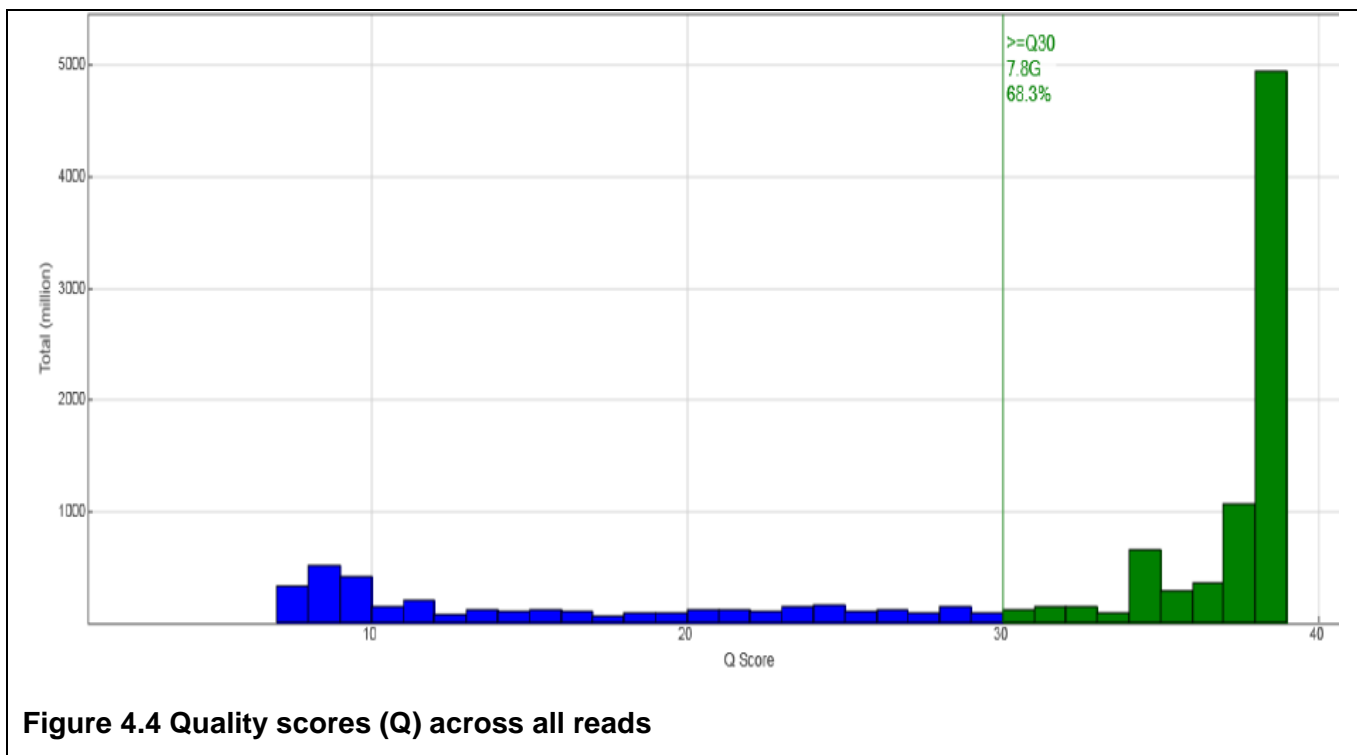


Figure 4.4 Quality scores (Q) across all reads

FastQC analysis showed that the sequence read lengths ranged between 35 and 301 bp. The average quality score per read was between 34 and 36 and the GC content ranged between 32 and 35%. No overrepresented sequences were found. The analysis indicated some sequence duplication in all of the samples; however this is most likely a false overrepresentation due to the long read length and high coverage of the sequences. The per base sequence content (representation of A, T, G or C) showed a deviation from normal distribution in the first nine bases of the reads; however, this is a known bias characteristic of Illumina sequencing and does not influence downstream analysis. The data were of sufficient quality to continue with sequence analysis.

4.3.4. Genome assembly and annotation

All but three isolates were assembled with the online SPAdes application through BaseSpace. The remaining three isolates consisted of more than 1.0 gigabases of data and were run with

SPAdes using command line options. Figure 4.5 below shows a QUAST quality report of one of the assemblies (TKB-106); and average quality results for selected statistics for all assemblies are shown in Table 4.3.

Assembly	contigs
# contigs (≥ 0 bp)	177
# contigs (≥ 1000 bp)	69
Total length (≥ 0 bp)	3067019
Total length (≥ 1000 bp)	3012696
# contigs	111
Largest contig	409715
Total length	3040687
GC (%)	32.67
N50	138062
N75	80405
L50	7
L75	14
# N's per 100 kbp	0

Figure 4.5 QUAST quality report (sample TKB-106). All statistics are based on contigs ≥ 500 bp, unless stated otherwise.

Table 4.3 Assembly statistics for all isolates

Assembly Statistic	Range (Min – Max)	Average
# contigs (≥ 1000 bp)	23 - 88	60
# contigs (≥ 500 bp)	44 - 113	81
GC (%)	32,12 – 32,9	32,68
N50	77590 - 243965	134350

As seen in Figure 4.5 and Table 4.3, the N50 values of all samples was >77500 and this indicates that the assemblies should have good quality. The number of contigs (≥ 500 bp) for the selected *S. aureus* isolates were between 44 - 113. The *S. epidermidis* isolate was expected to have a smaller genome than the *S. aureus* isolates and therefore had less than the average number of contigs ≥ 500 bp (64). The total length of each assembly was larger than the actual genome sequence, as many of the smaller contigs may have been misassemblies or regions already contained within larger assembled contigs. Mapping the assembly to a closely related reference helps to filter out these contigs, as a large percentage of them will remain unmapped and appear at the end of the alignment. This was visualized using ACT as shown in Figure 4.6.

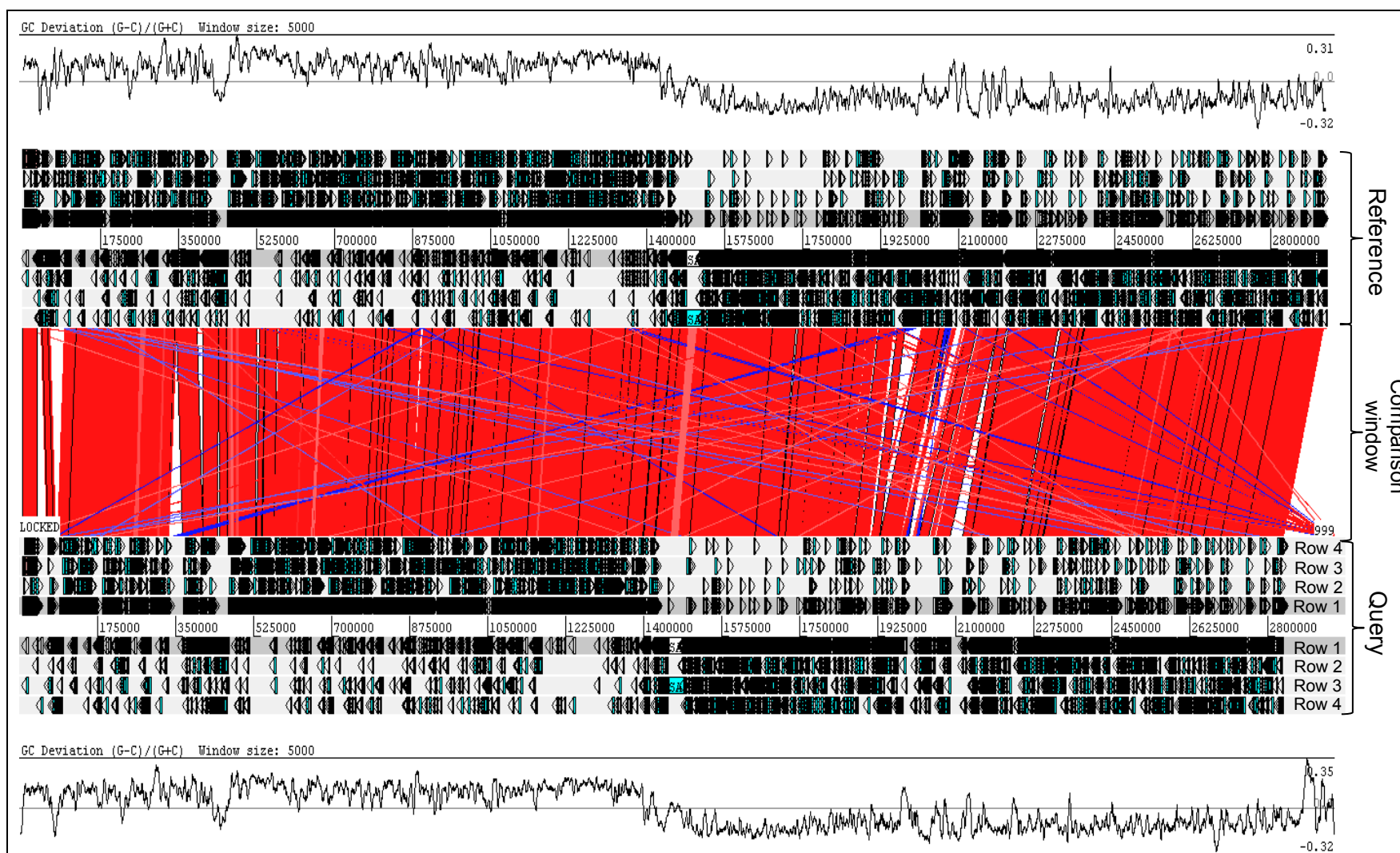
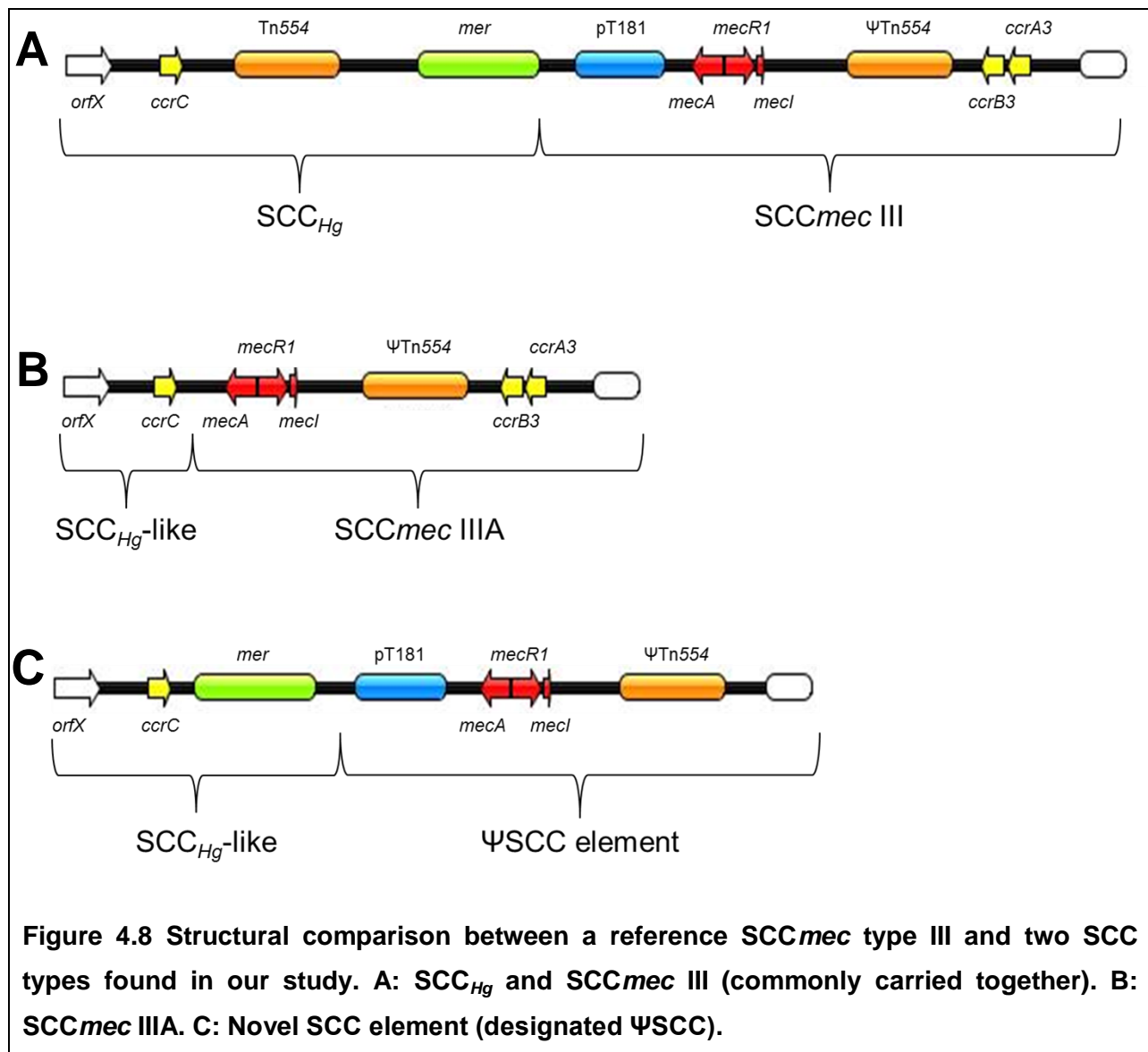


Figure 4.6 Visual comparison of a reference sequence (JKD 6008, top) and ordered contigs (TKB-105, bottom). Red blocks show similarity/synteny between the genomes, blue blocks show areas of synteny that have an inversed configuration in the query sequence. White areas are sequences that are unique to either the reference or query.

The ACT comparison (Figure 4.6) showed that the two genomes we aligned were highly similar, with isolated areas with unique sequences. The reference and query sequences can be found above and below the comparison panel respectively, with set intervals (in this case 175000 bp) marking the length of the genomes. For each genome, four rows extend from both the forward (top) and reverse strand (bottom), showing the relative position of all annotated features on the genome (row 1), as well as their position on either the first, second or third amino acid reading frame (rows 2 - 4). Two graphs were also included, showing the typical GC skew/deviation across the *S. aureus* reference genome and the query genome. Unmapped contigs, and those that mapped, but did not align well, were shown at the end of the query alignment (bottom). The GC skew for many of these unmapped contigs was extremely high, indicating that these regions may have a high copy number (e.g. repeat regions); and this may also explain why their alignment was unsuccessful. These contigs can be moved and ordered manually, however this requires advanced editing skills and is extremely laborious and time-consuming. The *SCCmec* region is found close to the origin of replication, and the alignment for this region is shown in Figure 4.7.

4.3.5. Novel SCC element structure

77



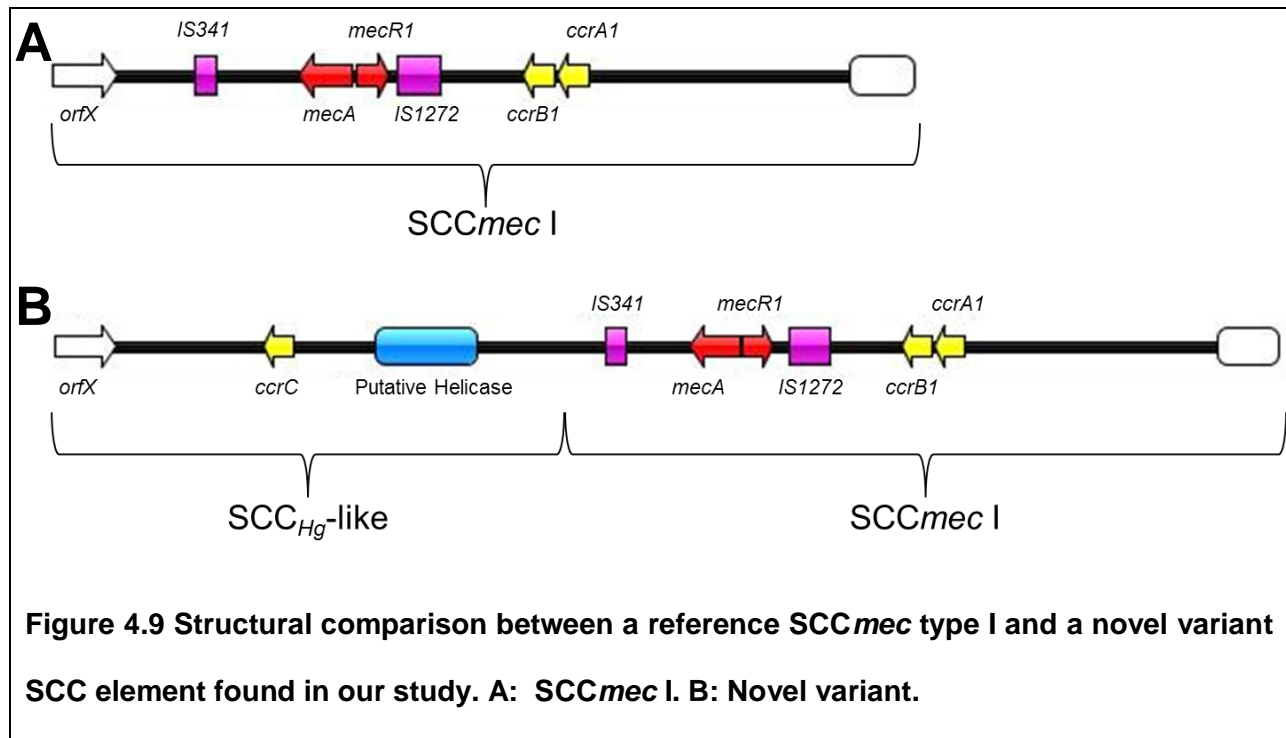
Our analysis showed that all of the *SCCmec* type III isolates that were submitted for sequencing carried *SCCmec* IIIA elements. Type IIIA elements have *mec* Class A in combination with *ccrA3ccrB3* (*ccr* Type 3). The lack of the pT181 tetracycline plasmid seen in our isolates is characteristic of *SCCmec* IIIA. ORFs typical of *SCC_{Hg}* were found between the J3 region and *orfX*, including *ccrC*. However, this region lacked the characteristic *Tn554* (erythromycin resistance) and *mer* (mercury resistance) regions found in complete *SCC_{Hg}* elements.

The novel SCC element was shown to carry the same *mec* complex as SCC*mec* type III (*mec* Class A), as well as some ORFs in the J2 region, namely the Ψ Tn554 region encoding for cadmium resistance. However, the element appeared to be truncated after the J2 region, downstream from Ψ Tn554, and was missing a functional *ccr* complex, as well as the majority of the J1 region. The truncated SCC-like element (designated Ψ SCC) was found together with a SCC_{Hg}-like element that lacked Tn554, but carried the *ccrC* and *mer* regions. The novel element also contained pT181, which is characteristic of SCC*mec* III, but not IIIA.

We were unable to visualize the structure of the novel element that we had postulated in our CoNS isolate, due to the lack of suitable reference strains carrying the appropriate SCC elements. However, we were able to identify key features and genes present in its genome. *orfX*, *ccrC* and *mec* Class A were all present and similar to those found in the novel SCC*mec* elements in *S. aureus*. With the exception of the tetracycline resistance plasmid, pT181, we found no other SCC-like resistance plasmids or transposons (Tn554 and Ψ Tn554) seen in the novel and IIIA SCC elements. A functional *ccr* complex type 4 (*ccrA4ccrB4*) was also present in this isolate.

4.3.6. Novel variant SCC element structure

When compared to SCC*mec* type I, the novel variant SCC element found in our study carried the same *ccr* type (*ccrA1ccrB1*) and the same *mec* (Class B) (Figure 4.9). The two insertion sequences, *IS341* and *IS1272* were found in the same direction and position relative to SCC*mec* type I. In addition to the SCC*mec* I regions, an additional SCC_{Hg}-like element was inserted between the J3 region and *orfX*. It is similar to the ones seen in our novel and SCC*mec* IIIA elements, but has a unique region showing 64% similarity to a putative staphylococcal superfamily II DNA/RNA helicase. No other resistance encoding transposons or plasmids were found.



4.4. Discussion

4.4.1. Characterization of the novel SCC element

We subjected a set of isolates representing the type III and novel SCC*mec* elements found in our study to whole genome sequence analysis in an attempt to confirm and clarify the results found with the SCC*mec* typing assays. Sequencing analysis confirmed that the isolates identified (by mPCR) as carrying SCC*mec* type III were indeed the variant IIIA (Chongtrakool et al. 2006) that lacked the pT181 plasmid. SCC*mec* typing showed that our type IIIA isolates also carried *ccrC*, indicating that they were possibly harbouring the SCC_{Hg} element; which is commonly carried together with SCC*mec* III (Ito et al. 2009). However, further typing failed to amplify any other SCC_{Hg} resistance elements. Whole genome sequencing showed that these isolates were carrying a truncated, SCC_{Hg}-like element harbouring *ccrC*, which integrated between *orfX* and the type IIIA J3 region. A truncated SCC_{Hg} element, Tn6072, has been described in the literature; however it only lacks the *mer* operon, whereas the one we described here lacks both *mer* and Tn554 (Chen et al. 2010).

Assembly of the novel SCC*mec* element confirmed the lack of type III *ccr* genes and J1 ORFs seen in the Milheirico (2007) and Kondo (2007) mPCRs; and showed a deletion of the *ccr* gene complex and the majority of the J1 region. A SCC_{Hg}-like element was also integrated between the J3 region and *orfX*. Although the novel element showed many similarities to our IIIA isolates, it also carried the pT181, encoding tetracycline resistance, found in SCC*mec* type III. The SCC_{Hg}-like element carried with the novel element was also different; as it contained the *mer* operon which was absent in the IIIA isolates. The novel element did not appear to carry a functional *ccr* complex, and would therefore be considered a ΨSCC element on its own. However, it is possible that the SCC_{Hg}-like element (carrying *ccrC*) has fused with the SCC element in a similar way as seen in SCC*mec* type VII (Berglund et al. 2008), thereby generating

a novel arrangement of the *ccr* and *mec* complexes. Further analysis is required to determine whether these elements have fused, or if they are a composite of two distinct SCC elements.

Although the initial SCC*mec* typing assays showed that the novel element in the MRSA isolates appeared similar to the SCC element in the CoNS isolate, genomic analysis showed a few key differences between the two. Unlike the novel SCC element in the MRSA isolates, the CoNS isolate did not carry the Ψ Tn554 or *mer* resistance regions. It was also shown to harbour a functional *ccr* complex (*ccrA4ccrB4*) which has previously been found in SCC-CI elements carried by *S. epidermidis* strains (Ito et al. 2009). These discrepancies in the SCC regions, as shown by genomic analysis, highlight the shortfalls of traditional SCC*mec* typing assays and show the importance of genome sequencing to assign SCC*mec* types accurately. Sequencing has the greatest discriminatory power for typing and is the gold standard for these assays. Even so, the cost of genomic analysis is too high for frequent use in resource limited settings and therefore SCC*mec* typing by mPCR still remains the suggested assay for epidemiological research (IWG-SCC 2016).

4.4.2. Characterization of the novel variant SCC element

As seen in the type IIIA and novel SCC elements, the novel variant SCC element also carried *ccrC* on a truncated SCC_{Hg}-like element. The SCC*mec* region of these isolates was almost identical to SCC*mec* type I and shared all of its main gene complex arrangements and ORFs. While the combination of SCC_{Hg} and SCC*mec* III is abundant, this is the first report to our knowledge of an SCC_{Hg}-like element carried together with a SCC*mec* type I element.

The main difference between the SCC_{Hg}-like element and the others found in our study is the presence of an ORF between *ccrC* and the SCC*mec* element, encoding a putative helicase. Helicases separate double stranded DNA into single strands and are essential for DNA replication. A BLAST search showed that this ORF only shared 64% similarity to its closest

match in an *S. epidermidis* genome and it may therefore represent a novel type helicase. It is not uncommon to find genes associated with replication in SCC elements and it has recently been suggested that these genes allow the SCC elements to replicate independently once they are excised from a genome, thereby increasing the efficiency of HGT (Mir-Sanchis et al. 2016).

4.4.3. Dissemination of the novel SCC and SCC_{Hg}-like elements in our setting

Our results show that truncated SCC_{Hg}-like elements were widely disseminated in our setting and were found in the majority of our isolates, including the novel, novel variant and SCC_{mec} IIIA isolates. It has been suggested that the spread of smaller or truncated elements may confer a fitness advantage to the population; however further research needs to be done to assess fitness benefits (Boyle-Vavra & Daum 2007; Chen et al. 2010). A second unconfirmed explanation may be that smaller elements such as the ones seen in our setting are much easier to excise and integrate into genomes, and therefore spread more easily than the larger elements.

Although we did not find the novel SCC element in our CoNS population, research has suggested that the SCC_{Hg} element originated from CoNS and was transferred to *S. aureus* (Chongtrakool et al. 2006). While there is a need to compare the differences and similarities of SCC elements between *S. aureus* and CoNS isolates; working with CoNS genomic data presents significant difficulties. The lack of reference sequences and standardization of SCC_{mec} types in CoNS and the sheer genetic diversity and variety of species in this group are major obstacles in this field of research (Zong et al. 2011). This held true in our own setting where we were able to identify the presence of individual elements, but unable to fully construct the novel SCC region in our sequenced CoNS isolate, even when aligned to a suitable reference. In order to construct the entire element, primers will need to be designed for targeted sequencing of long range PCR products spanning the length of the element.

4.4.4. Conclusion

Preliminary genomic analysis showed that the presence of *ccrC* in our novel and novel variant SCC elements was conveyed by structures similar to SCC_{Hg} . In both cases the SCC_{Hg} -like element was truncated and did not carry all of the genes it is traditionally associated with. The novel SCC element did not appear to carry a functional *ccr* complex, but it is possible that there was integration between it and the SCC_{Hg} -like element. Further analysis is therefore necessary to confirm whether the novel and novel variant SCC elements found in our setting are composites of multiple SCC elements, or if the rearrangements in structure constitute novel SCC_{mec} types. The International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) will be contacted to assist in the classification of these elements.

The dissemination of the novel SCC element among local MRSA isolates may reflect clonal spread within the hospital. The finding of a similar novel element within a MR-CoNS isolate suggests CoNS as a potential source, however further phylogenetic analysis is required to provide insights into how these novel elements emerged.

During this study, we produced large amounts of genomic data that will be vital for future research. In-depth analysis of the SCC and other resistance regions will provide insights into the characteristics of our MRSA population. Phylogenetic analysis using WGS data provides the highest level of discrimination and will accurately show the relationship of our local MRSA isolates with global strains. The genome drafts will be submitted to the NCBI database once they have been fully curated and annotated.

Chapter 5: Conclusion

Methicillin resistance in staphylococcal species, both *S. aureus* and CoNS, can be largely attributed to the acquisition of the large mobile genetic element (MGE), *SCCmec*. *SCCmec* elements consist of two major gene complexes, *mec* and *ccr* and various other resistance determining factors. Many non-*mec* SCC elements have also been discovered and are common in MR-CoNS isolates.

In this study, we aimed to describe the epidemiology of *SCCmec* types present in MRSA and MR-CoNS isolates from Tygerberg Hospital. We utilized a mPCR-based *SCCmec* typing assay developed by Milheirico et al. (2007) to determine the prevalence of the major healthcare associated *SCCmec* types in the MRSA and MR-CoNS populations. Typing showed that the majority of the MRSA population belonged to either a putative novel *SCCmec* type or a novel variant *SCCmec* type; however only one novel type was identified in the MR-CoNS group. Furthermore, the MR-CoNS population consisted of a large percentage of *SCCmec* IV, but the majority were not typeable by conventional *SCCmec* typing schemes. The lack of the novel element in the MR-CoNS population could be an indication that it conveyed no evolutionary advantage to the population. While it is unlikely, differences in collection time of the two populations may have influenced the results and may therefore be useful to screen the current MRSA population, to confirm that the novel and novel variant *SCCmec* elements are still present.

We investigated the novel and novel variant SCC elements in more detail, by performing a comprehensive typing assay described by Kondo et al. (2007) on a subset of representative isolates. This protocol confirmed the results from the Milheirico (2007) protocol, but also provided a more descriptive picture of additional resistance determinants present in the novel and novel variant SCC elements. The novel, novel variant and representative isolates from *SCCmec* type III were shown to carry *ccrC*, a gene commonly found on an SCC element known

as SCC_{Hg}. In contrast, none of these isolates showed amplification for a resistance determining transposon, Tn554, which is carried on SCC_{Hg}. While PCR-based SCC_{mec} typing can determine the presence or absence of multiple genetic targets, a major drawback is that their position and relative orientation to one another is not revealed. To combat this, novel SCC_{mec} types need to be characterized by sequencing the entire length of the element.

We selected representative isolates from our study population for whole genome sequencing using the NGS Illumina platform. Using this data, we constructed the SCC_{mec} elements of the novel, novel variant and SCC_{mec} type III isolates and compared the structures to those of closely related reference strains. The novel variant was shown to be a possible composite of two SCC elements, SCC_{mec} I and a truncated SCC_{Hg} element. Our type III isolates were shown to be IIIA variants that do not carry pT181 resistance determining plasmids, but a truncated SCC_{Hg} element could also be found between the 5' end of the element and the SCC_{mec} IIIA *mec* complex. The novel element was very similar to SCC_{mec} type III, but it was shown to be truncated and did not contain a functional *ccr* complex. A SCC_{Hg}-like element encoding a mercury resistance operon was present between *orfX* and the pT181 plasmid. It is possible that some of the composite elements found in this study have integrated with their respective SCC elements and are effectively carried together. If this is truly the case, it may constitute a novel SCC_{mec} element; however further research is necessary to investigate whether integration has taken place.

Future directions

The novel and novel variant SCC structures described in this study need to be confirmed using in-depth genome analysis. This may include the use of PCRs designed to confirm the presence and position of key integration sequences in and around the elements and to define the start and end of the elements. This will enable us to determine whether the novel and novel variants

are composites of two elements, or if integration has taken place and constitute novel SCC*mec* types. We will consult with the IWG-SCC on the naming and classification of these elements.

The whole genome sequencing data is a great store of information and has multiple uses for future research. This may include searching for other antibiotic resistance genes and factors, virulence factors and phylogenetic characteristics. This data will enable us to compare our isolates to those found locally and globally and give insights into the origin of these clones in our setting.

Addendum A: Materials

A1 Buffers and media

Table A1: Buffer compositions

Media / Buffer	Composition
DNA extraction buffer	5% monosodium glutamate; 50 mM Tris-HCl pH 7.4; 25 mM EDTA
Proteinase K buffer	5% SDS; 100 mM Tris-HCl pH 7.8; 50 mM EDTA
TAE	40mM Tris base; 20mM acetic acid; 1mM EDTA
TE	10 mM Tris-Hcl pH 8.0; 1 mM EDTA

A1.1 Tryptic Soy Broth (TSB) preparation

15 g Agar powder and 15 g TSB medium were added to a glass container and filled up to 1 L with millipore distilled water. The mixture was placed on a stirring heating block until dissolved and was then autoclaved for 15 minutes at 121°C to sterilize the media. Once cool to touch, the media was cast in sterile plates, allowed to set and stored at 4°C.

A2 Gel electrophoresis

A2.1 Agarose gels

2%: 2 g of SeaKem LE Agarose powder (Lonza) was dissolved in 100 mL 1X Tris-acetate-EDTA (TAE) at high heat. The composition of the TAE buffer is shown in Table A1. Gels were cast and allowed to set for at least 30 minutes prior to use.

A2.2 DNA ladders (size markers)

The KAPA Universal DNA Ladder (KAPA Biosystems) was used for band size comparison in the Milheirico (2007) and Kondo (2007) protocols.

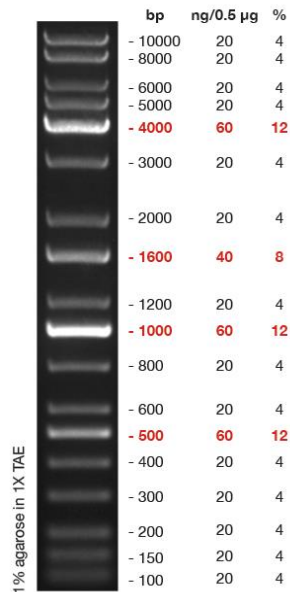


Figure A1: KAPA Universal DNA Ladder

A2.3 Loading dye

NovelJuice (GeneDireX) was used as a loading dye and is a non-toxic, non-mutagenic fluorescent alternative to ethidium bromide staining. It contains three tracking dyes (Xylene Cyanol FF, Bromophenol Blue, and Orange G) for visually tracking DNA migration during the electrophoresis process.

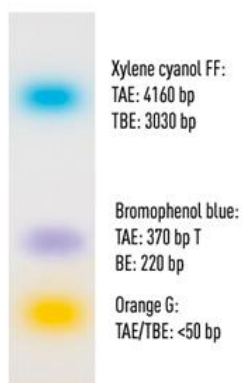
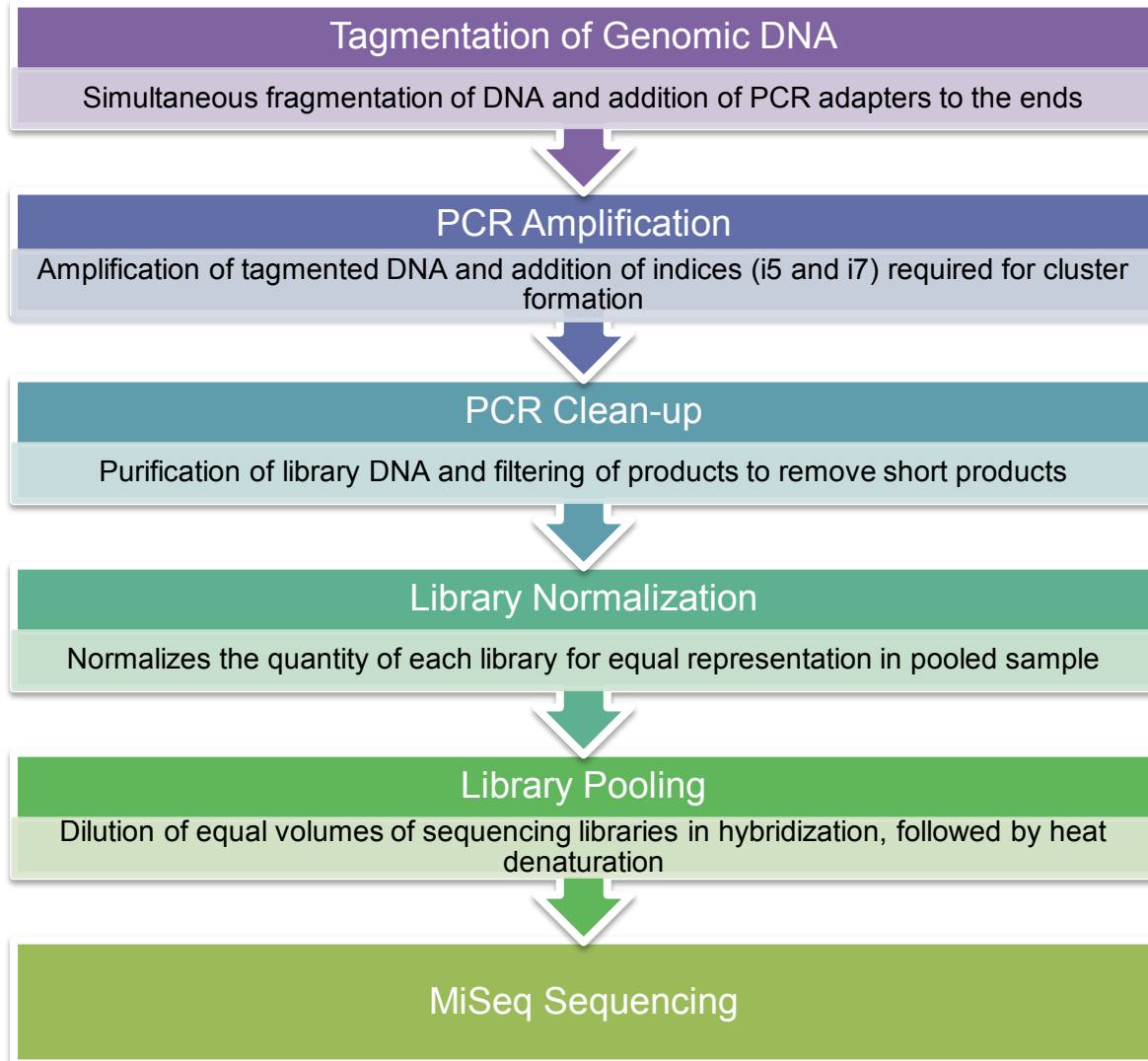


Figure A2: NovelJuice Tracking Dyes

Addendum B: Supplementary Methods

B1 Nextera XT DNA Sample Preparation Workflow (Illumina 2016)



B2 Genome assembly and annotation commands

The specific parameters used during the genome assembly and annotation process are listed below in grey blocks, preceded by a command description marked with the # symbol.

```
# Genome alignment (makeblastdb): A BLAST comparison database was generated using the reference sequence with the tool makeblastdb
```

```
The database type needs to be specified as being DNA (not protein) by entering the parameter: -dbtype nucl
```

```
# Genome alignment (blastn): A BLAST search was done using the blastn tool, to compare the query sequence with the reference sequence.
```

```
The type of BLAST search needs to be specified as nucleotide to nucleotide:
```

```
-task blastn
```

```
The comparison is best viewed in alignment output type 6:
```

```
-outfmt 6
```

```
# Genome annotation (RATT): Annotation was transferred between two assemblies (one reference, one query)
```

```
The type of annotation transfer needs to be specified as Assembly
```

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